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MECHANISMS OF THERAPEUTIC RESISTANCE IN CASTRATION RESISTANT PROSTATE CANCER

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MECHANISMS OF THERAPEUTIC RESISTANCE IN CASTRATION RESISTANT PROSTATE CANCER

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

MECHANISMS OF THERAPEUTIC RESISTANCE IN
CASTRATION RESISTANT PROSTATE CANCER

Taxane based chemotherapy is an effective treatment for castration-resistant prostate cancer (CRPC) via stabilization of microtubules. Progression to castration-resistant prostate cancer is characterized by increased androgen receptor (AR), elevated intra-prostatic androgens and activated AR signaling despite castrate levels of androgens.

Previous studies identified that the inhibitory effect of microtubule targeting chemotherapy on AR activity was conferred by interfering with AR intracellular trafficking. The N-terminal domain (NTD) of AR was identified as a tubulin interacting domain that can be effectively targeted by the novel small molecular inhibitor, EPI. Taken together, this evidence provided the rationale that targeting AR nuclear translocation and activity via a combination of an antagonist of the AR NTD and taxane based chemotherapy may enhance the therapeutic response in CRPC. This study investigated the anti-tumor efficacy of a combination of EPI with Docetaxel chemotherapy, in cell models of CRPC, harboring AR splice variants in addition to full length AR. Our findings demonstrate there was no significant effect on androgen-mediated nuclear transport of AR variants and transcriptional activity by Docetaxel. The therapeutic response to Docetaxel was enhanced by inhibition of the NTD of AR (by EPI) through cycling of epithelial-mesenchymal transition (EMT) to mesenchymal–epithelial transition (MET) among prostate cancer epithelial cells. These results support that transient programming of EMT by the AR NTD inhibitor, potentially drives sensitivity of prostate tumors with differential distribution of AR variants to microtubule targeting chemotherapy.

The FDA-approved next-generation taxane, Cabazitaxel (CBZ) and the anti-androgen Enzalutamide (MDV3100) have demonstrated additional survival benefits for patients with advanced CRPC. The present study pursued the mechanism of therapeutic resistance to Cabazitaxel and anti-androgen treatment *in vitro* and *in vivo* models of CRPC. The findings support the ability of Cabazitaxel to target pro-mitotic kinesins, providing an insight into a new mechanism of action of this chemotherapy. Moreover, the in vivo evidence identified that Cabazitaxel chemotherapy has novel effects beyond suppressing tumor growth, by inducing prostate glandular differentiation. Our results are of translational significance in introducing a novel mechanism for cross-resistance to Cabazitaxel chemotherapy and anti-androgen therapy and a potential targeting platform to overcome such resistance in advanced CRPC.

KEYWORDS: Prostate Cancer, Androgen Receptor,
Microtubules, Taxanes, Anti-Androgens

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MECHANISMS OF THERAPEUTIC RESISTANCE
IN CASTRATION RESISTANT PROSTATE CANCER

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Chapter 1. Introduction:

The Clinical Challenge for Treatment of Advanced Prostate Cancer

Over seventy years ago, male steroid hormones were implicated in prostate cancer proliferation by Huggins and Hodges (1941), and withdrawal of these hormones was shown to diminish this growth (Huggins & Hodges, 1941). Androgen deprivation therapy (ADT) can be achieved by a variety of surgical and/or pharmacological methods, but regrettably ultimately, fails to effectively cure patients with prostate cancer. Castration resistant prostate cancer (CRPC) occurs when prostate tumor cells begin to proliferate again in the absence of male steroid hormones and this represents a much more aggressive disease state. Biochemical recurrence can be monitored in patients by sequential evaluation of serum prostate specific antigen (PSA); nearly 70,000 American men develop this biochemical recurrence per year (Freedland & Moul, 2007; Hu, Denmeade, & Luo, 2010). PSA screening facilitates the identification of at risk patients experiencing biochemical recurrence to metastatic CRPC progression. Progression to CRPC is characterized by increased AR expression in the prostate tissues and perpetual AR signaling despite physiological castrate levels of androgens (C. D. Chen et al., 2004; B.J. Feldman & D. Feldman, 2001). Localized prostate cancer is highly curable with radical prostatectomy or radiation therapy, but among men who progress to CRPC, median survival is less than two years and 90% of these men will endure bone metastases (Hotte & Saad, 2010). Until very recently, taxanes were the only chemotherapy class to confer additional survival and palliative benefit to CRPC patients. Over-expression and localization of the AR to the nucleus has been associated with reactivation of the androgenic signaling axis and progression to metastatic CRPC in patients (B. J. Feldman & D. Feldman, 2001).

Taxanes are cytotoxic chemotherapeutic agents which bind to the β tubulin subunit of the protofilament of the microtubule, stabilizing the structure of the cellular cytoskeleton. This stabilization prevents cells from dividing, inducing mitotic arrest and apoptosis. While the antimitotic capability of taxanes is irrefutable, prostate cancer tumors are not particularly fast growing. In fact, prostate tumor cells have characteristically slow doubling times in patients suggesting that taxanes are affecting more than just mitotic events. The goal of this project was to dissect the mechanisms and molecular events of taxane treatment beyond the antimitotic effects in tumors resistant to anti-androgen therapies and their exploitation towards optimal therapies for patients with metastatic CRPC.

Androgen Receptor Signaling

The role of the androgen receptor (AR) signaling axis in the progression of prostate cancer is a cornerstone to our current understanding of the molecular mechanisms behind this malignancy that has become an American epidemic. The cross talk of AR with other critical signaling pathways may explain the advancement of prostate cancer to metastatic castration resistant prostate cancer (CRPC) (M.-L. Zhu & Kyprianou, 2008). Of particular interest to such crosstalk are the pathways associated with epithelial to mesenchymal transition (EMT). Reactivation of EMT is a hallmark of metastatic cancer spread, and recent evidence suggests the involvement of AR in the signaling pathways regulating EMT (M. Zhu & Kyprianou, 2010). Cadherin switching, EMT inducing transcription factors, Wnt, TGF-β, and Notch signaling can all be modulated by crosstalk with the AR. The current understanding of the functional exchanges between the androgen signaling by AR activity and key growth factor signaling pathways that impact EMT towards prostate cancer progression to metastatic

CRPC is discussed towards appreciation of the clinical relevance in the effective targeting of advanced disease.

The AR is a member of the steroid-thyroid-retinoid nuclear receptor superfamily found on the X chromosome (Xq11-12) spanning approximately 180kb of DNA with 8 exons (Gelmann, 2002). In normal AR signaling, testosterone synthesized in the testis or adrenal gland is sequestered by sex hormone binding protein (SHBP) circulating in the blood. Testosterone dissociates from SHBP and diffuses across the plasma membrane, bringing testosterone into close proximity with 5 α- reductase (SRD5A1, SRD5A2) (cytochrome p450 enzyme) producing the cognate ligand of AR: dihydrotestosterone; DHT (Lonergan & Tindall, 2011; Schmidt & Tindall, 2011; Wilson, 2001). When AR binds its cognate ligand DHT, it facilitates the rearrangement of AR protein domains, facilitates conformational change within the heat shock protein 90 (Hsp90) super complex and subsequent transcriptional activation after translocation. DHT bound AR undergoes homo-dimerization and phosphorylation by the Protein Kinase A signaling pathway resulting in activation (Brinkmann et al., 1999; Nazareth & Weigel, 1996). The AR homo-dimers translocate to the cellular nucleus and may bind androgen responsive genes (ARG) at specific palindromic DNA sequences known as androgen responsive elements (ARE) (Feng, Zheng, Wennuan, Isaacs, & Xu, 2011). Upon binding to ARE, AR dimers can act as a scaffold towards the recruitment of accessory proteins to assemble an active transcription complex (Feng et al., 2011; Heinlein & Chang, 2002; Roy, Lavrosky, & Song, 1999). AR binding to ARE forms a stable pre-initiation complex near the transcriptional start site facilitating the recruitment and initiation of RNA polymerase II (Figure. 1.2.B) (Jenster, van der Korput, Trapman, & Brinkmann, 1995).

Structurally, AR is composed of an amino-terminal activating domain (NTD), a carboxy-terminal ligand binding domain (LBD), a DNA binding domain in the mid-region that contains two zinc finger motifs to facilitate the interaction of the protein with the DNA double helix (DBD), and a hinge region to facilitate the change in protein folding upon binding to the ligand and dimerization (Figure. 1.1.A). These four domains comprise the 919 amino acid protein with a mass of 110 kDa. N-terminal Domain: The NTD (exon 1, amino acids 1-537) has been shown to possess multiple transcriptional activating units (TAU): TAU-1 and TAU-5 (Figure. 1.1.A) (Jenster et al., 1995). TAU-1 is associated with wild type AR transcriptional activation, and is characterized by a high number of acidic amino acids, three glutamine repeats, and a phosphorylation site. Conversely, the TAU-5 sequence is characterized by stretches of proline, alanine, and glycine (Jenster et al., 1995). TAU-5 is responsible for the constitutive transcriptional activity of the NTD and is mediated by a core sequence of 435WHTLF439 in between the aforementioned alanine and glycine stretches (Figure 1.1.A) (Dehm, Regan, Schmidt, & Tindall, 1989; Lonergan & Tindall, 2011). The cysteine rich DBD (exons 2 and 3, amino acid: 68) contains two important motifs (Simental, Sar, Lane, French, & Wilson, 1991). The P-box motif found in the first of two zinc fingers facilitates the interaction of the AR with gene specific nucleotide sequences inside the major groove of the DNA double helix (Figure. 1.1.A) (Umesono & Evans, 1989). The D-box motif mediates the DBD/LBD interaction that allows for inter-domain interaction and AR homo-dimerization after activation and facilitates the spacing of the AR over the half sites and binding on the ARE (Fig. 1.1.A) (Lonergan & Tindall, 2011; Umesono & Evans, 1989; van Royen, van Cappellen, de Vos, Houtsmuller, & Trapman, 2012; Zhou, Sar, Simental, Lane, & Wilson, 1994).The DBD contains one of the nuclear localization signals (NLS) as discussed below.

With only approximately 50 amino acids, the hinge region packs a big punch in a small space. The flexible hinge region separates the LBD and the DBD while containing part of the bipartite nuclear localization signal (Figure. 1.1.A) (Haelens, Tanner, Denayer, Callewaert, & Claessens, 2007; Robinson-Rechavi, Escriva, & Laudet, 2003). The hinge region (as well as DBD and LBD) also contains a site for interaction with Filamin A, an actin interacting protein and signaling scaffold required for nuclear translocation of the AR (Lonergan & Tindall, 2011; Ozanne et al., 2000). The hinge region plays a role in nuclear localization, DNA binding inhibition, coactivator recruitment, and the N terminal / C terminal interaction of the AR (Haelens et al., 2007). A span of highly basic residues between 629 and 636 (629-RKLKKLGN-636) is conserved in all AR sequences known and decreases the affinity of AR for DNA binding as demonstrated by deletion constructs (Haelens et al., 2007). Ligand Binding Domain: The LBD (exons 4-8, ~250 amino acids) mediates the binding of the AR ligand (testosterone or DHT) to the AR protein and initiates the downstream cascade of the androgen signaling axis (Lonergan & Tindall, 2011). In addition to ligand binding, the LBD associates with the heat shock protein super-complex, interacts with numerous co-regulators and participates in receptor dimerization (Figure. 1.1.A and Figure 1.1.B) (McKenna, Lanz, & O'Malley, 1999; Poukka et al., 2000; Pratt & Toft, 1997; Xu, Glass, & Rosenfeld, 1999). The AR protein is composed of two activation domains (AF-1 and AF-2). The AF-1 domain is localized to the NTD and is composed of TAU-1 and TAU-5 domains contributing to the transcriptional activation program (Figure 1.1.A). The AF-2 domain is localized to the LBD and interacts with LxxLL-containing co-regulators such as the steroid receptor co-activators [SRC], and TAU domains in the NTD (Heery, Kalkhoven, Hoare, & Parker, 1997; Lonergan & Tindall, 2011).

Nuclear translocation of the AR is inhibited by its sequestration in the Hsp90 super-complex, tethering it to the cytoskeleton. The nuclear localization signal (NLS1) of AR is bipartite and spans the DBD and hinge regions of the protein with exons 3 and 4 represented (Zhou et al., 1994). Nuclear translocation of the full length AR is protected by the bipartite nature of the NLS; this feature safeguards that cooperation between the domains occur before nuclear transport may proceed (Zhou et al., 1994). Binding of AR to its cognate ligand facilitates a conformational change that brings the NLS into a functional orientation for signaling translocation (Zhou et al., 1994). After nuclear translocation, NLS allows the binding of AR to the importin-α adaptor protein and importin-β carrier protein (S. C. Chan, Y. Li, & S.M. Dehm, 2012), facilitating movement through the nuclear pore complex and Ran-dependent release into the nucleus (Black & Paschal, 2004; Brodsky & Silver, 1999; Corbett & Silver, 1997; Cutress, Whitaker, Mills, Stewart, & Neal, 2008; Gorlich, 1997; Nigg, 1997). A second NLS sequence (NLS2) exists in the LBD and allows AR to enter the nucleus via importin-α independent mechanism (Picard & Yamamoto, 1987; Poukka et al., 2000; Savory et al., 1999). In the cytosol, the hinge region of AR and the Hsp90 super-complex mediates interaction with the 280kDa cytoskeletal protein, Filamin-A (FLNA) (C. J. Loy, Sim, & Yong, 2003; Ozanne et al., 2000). Filamin-A is an important regulator of the solation-gelation equilibrium at the cell membrane. By cross-linking F-actin fibers into orthogonal arrays, it is able to affect the stability of the cytoskeleton that lies beneath the cell membrane (Koteliansky, Shirinsky, Gneushev, & Smirnov, 1981; Ozanne et al., 2000). Filamin-A contributes to mediating the translocation of the AR to the nucleus upon activation by facilitating interaction with the microtubule associated motor protein, dynein (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, X.K., et al., 2011; C. J. Loy, Sim, K.S., Yong, E.L., 2003; Ozanne et al., 2000).

Various co-regulatory proteins interact with AR and have been expertly reviewed elsewhere (Heemers & Tidall, 2007). Of particular interest are the intra-molecular interactions within the AR protein domains and between AR subunits in a homo-dimeric complex which are of utmost importance to the activation and nuclear translocation (van Royen et al., 2012). Upon binding DHT, the D-box of the DBD interacts with the TAU-1 domain of the NTD, an N-terminal to C-terminal protein domain interaction that is initiated in the cytoplasm (Schaufele et al., 2005; van Royen et al., 2012). The interaction between the D-box and the NTD of the AR is essential for the transition towards inter-AR molecule homo-dimerization (Schaufele et al., 2005). Homo-dimerization occurs in the cytosol prior to reaching the nucleus. Upon binding DNA, the N-terminal/C-terminal intramolecular interaction is finished freeing these domains to interact with AR co-regulators and the homo-dimer settles into the major groove of the DNA double helix (Van Royen et al., 2007; van Royen et al., 2012).

Epithelial-Mesenchymal Transition (EMT) in Prostate Cancer Progression

The biological process of EMT was first described in the context of normal organ development (Greenburg & Hay, 1982). Reactivation of EMT quickly became a hallmark of metastatic tumors. EMT is observed extensively in non-pathological conditions such as mechanisms of development including gastrulation and neural crest development in which epithelial cells must de-differentiate to a mesenchymal form, migrate, and re-differentiate into a new structure or organization (Yilmaz & Christophori, 2009). EMT can be classified into three distinct subtypes based on the biological setting hosting its manifestation (Kalluri & Weinberg, 2009). Type 1 EMTs are associated with embryonic

implantation and gastrulation facilitating the stratification of the germinal layers (Kalluri & Weinberg, 2009). Unlike Type 1, Type 2 EMTs are associated with wound healing, tissue regeneration, and organ fibrosis. Type 2 EMTs are characteristically induced by inflammatory signaling, either as a response to injury-induced inflammation as seen in wound healing or ongoing inflammation of certain organs resulting in fibrosis. Type 3 EMTs occur in neoplastic cells that undergo a manifold of genetic or epigenetic changes resulting in localized tumor cell proliferation. The Type 3 EMT is responsible for changes that facilitate tumor cell invasion and metastasis (Kalluri & Weinberg, 2009).

Emerging evidence suggests that reactivation of EMT processes may facilitate the development of prostate cancer (Matuszak & Kyprianou, 2011b) , with increasing number of studies focusing on the direct involvement of androgen/AR signaling in EMT / MET transitions. The clinic-pathological significance of EMT in human cancers continues to be a topic of debate. EMT is being interrogated by proteomic analysis towards defining its role in prostate cancer progression to metastasis. Investigating the regulatory mechanisms by which EMT programs are controlled by the androgen/AR signaling, is fundamentally important for understanding the functional contribution of EMT to various stages of prostate tumor progression to metastatic CRPC disease and emergence of therapeutic failure.

Mechanistically, EMT can be activated by TGF-β and receptor tyrosine kinase (RTK) / Ras signaling in addition to the well-known canonical Wnt-/B-catenin, Notch, Hedgehog, and NFκB dependent pathways (Huber, Kraut, & Beug, 2005). “Cadherin switching” is an important regulatory step in EMT induction, regulated by transcriptional regulators including Snail and Twist (Huber et al., 2005). Recent investigations from this laboratory strongly implicate the androgen signaling axis as an active participant in the progression of the mechanistic sequelae of EMT (Matuszak & Kyprianou, 2011b; M. Zhu

& Kyprianou, 2010). In what is seemingly becoming a controversial topic, androgens can induce EMT-associated changes in prostate cancer cells, regardless of their androgen sensitivity, conferring enhanced invasive and motile capacity via modulating the transcriptional regulator, Snail (M. Zhu & Kyprianou, 2010). Moreover, an inverse relationship between AR expression and extent of androgen-induced EMT induction suggest that very low level AR expression such as that seen immediately after beginning ADT may be contributing to metastatic spread of prostate cancer tumor cells (M. Zhu & Kyprianou, 2010). Studies by other investigators have shown that prostate cancer cells expressing AR in androgen-deprived conditions undergo an EMT, characterized by decreased E-cadherin and increased N-cadherin and Vimentin (Y. Sun et al., 2011). Increased N-cadherin expression and metastasis was seen in LNCaP xenografts and human clinical specimens (Tanaka et al., 2010). The β-catenin/ Wnt dependent signaling pathway is already a well-known accomplice in progression to EMT and metastasis, but the implication of this pathway under androgenic drive is essential to understanding prostate tumor specific EMT. Recent exciting insights into EMT regulation in prostate cancer implicates β-catenin in the androgen modulated EMT effect (M. Zhu & Kyprianou, 2010).

The physiological phenomenon known as “cadherin switching” has been accepted as a hallmark of EMT. E-cadherin or epithelial cadherin is an important cell adhesion protein mediating intercellular contacts and facilitating maintenance of tissue architecture. This is a protein essential to formation of adherens junctions which in combination with tight junctions mediate intercellular adhesion (Yilmaz & Christophori, 2009). E-cadherin is structurally characterized as a single pass transmembrane glycoprotein which forms calcium dependent homotypic interactions with E-cadherin on cell neighbors (Yilmaz & Christophori, 2009) These interactions are anchored to the

cytoskeleton by interactions with microfilaments composed of actin and mediated by β-catenin and α-catenin (Yilmaz & Christophori, 2009). E-cadherin expression can be lost, nonpolar, or expressed in the cytoplasm or alternatively transcriptional repression of E-cadherin can occur by diverse mechanisms engaging AR and its transcriptional co-regulators (Nelson & Nusse, 2004). Loss of E-cadherin expression results in loss of normal cell-cell interactions and facilitates progression of EMT and leads to metastasis (Harris & Tepass, 2010; Huber et al., 2005; Thiery, 2002; Thiery, Acloque, Huang, & Nieto, 2009. Upon E-cadherin loss, N-cadherin expression is enhanced to promote the mesenchymal cell phenotype. N-cadherin or Neural-cadherin is a mesenchymal cell association protein that allows transient cell-cell contacts typically expressed in cell types including smooth muscle, myofibroblasts, endothelial cells, neurons, and neoplastic cells {Matuszak, 2011 #210; Yilmaz & Christophori, 2009). The cell types usually expressing N-cadherin are also typical components of the reactive stroma composing the microenvironment of the prostate cancer tumor cell (Yilmaz & Christophori, 2009). The interactive mode employed by N-cadherin is not unlike that used by E-cadherin, this single span, transmembrane protein engages in homotypic interactions with N-cadherin on neighboring cells. Loss of E-cadherin has been associated with increasing Gleason grade in prostate cancer and the concept of cadherin switching is traditionally considered as predictive of metastatic development (Gravdal, Halvorsen, Haukaas, & Akslen, 2007; Jeanes, Gottardi, & Yap, 2008).

As illustrated in Figure 1.2.A, E-cadherin expression is repressed by the zinc-finger transcription factor Snail (SNAI1) (Acloque, Adams, Fishwick, Bronner- Fraser, & Nieto, 2009). Snail gained notoriety as a master regulator of EMT induction, but also plays an essential role in embryonic development and cell survival. Snail employs a mechanism of action whereby the transcription factor binds to the E-box of the E-

cadherin promoter and silences gene expression promoting a mesenchymal phenotype (Figure. 1.2.B) (Matuszak & Kyprianou, 2011b). Interestingly enough, Snail is functionally capable of modulating expression of proteins involved in tight junctions, including claudins, occludins, mucin-1 and cytokeratin 18 (Baritaki et al., 2009). Further fulfilling its infamy of "master regulator," Snail increases expression of mesenchymal phenotype associated markers and proteins associated with invasive capacity: vimentin, fibronectin, metalloproteinase-2, -9, ZEB1, and LEF-1 (Baritaki et al., 2009). To dissect the functional contribution of Snail to prostate cancer cell EMT, one must focus on its crosstalk with the AR signaling axis. Indeed, AR may function in an analogous manner to Snail, thereby repressing the expression of E-cadherin and promoting EMT by itself (Figure 1.2.B) (Y. N. Liu, Liu, Lee, Hsu, & Chen, 2008). Work from this laboratory has demonstrated that in an androgen-responsive, TGF-β-responsive, prostate cancer cell line, expression of Snail is significantly increased by exposure to DHT alone or in combination with TGF-β (Figure 1.2.B) (M. Zhu & Kyprianou, 2010). These observations support a functional involvement of the AR signaling navigated by Snail in acquisition of EMT characteristics of prostate tumor cells towards metastatic progression. Recent high throughput DNA analyses have furthered this investigation at the molecular level by identifying an ARE/ARG in the promoter region of Snail2 (slug), suggesting the direct modulation of Snail2 by AR (Bolton et al., 2007).

Several transcription factors regulate the expression and transcriptional activation of genes controlling the EMT phenomenon. Identification of those which specifically interact with the AR signaling axis provides a unique molecular platform begging exploration in prostate cancer (Figure. 1.2.B). Zeb1 (ZFHX1a gene) and Zeb2 (ZFHX1b) are closely related transcription factors whose activity has been strongly implicated in EMT (Anose & Sanders, 2011). These transcription factors are

characterized by separated clusters of Zinc finger domains (7 total) which recognize the CAGGTA/G E-box promoter element (Brabletz & Brabletz, 2010). ZEB1 modulates diverse-function genes. It significantly contributes to EMT by repression of E-cadherin expression, genes encoding basement membrane components, and regulators of cell polarity. Other effects run the spectrum from tumor suppression to anti-adipose accumulation *in vivo* (Anose & Sanders, 2011; Brabletz & Brabletz, 2010; Hidaka, Nakahata, & Hatakeyamam, 2008; Matuszak & Kyprianou, 2011b; Saykally, Dogan, Cleary, & Sanders, 2009). Progression to metastasis is an event mediated by ZEB1, in addition to its important involvement in facilitating trans-endothelial migration (Drake, Strohbein, Bair, Moreland, & Henry, 2009; Spaderna et al., 2008). Clearly, ZEB1 plays an important role in orchestrating complex physiological processes such as, but certainly not limited to EMT. Recent work has revealed a bidirectional negative feedback loop between AR and Zeb1 that has implicated ADT in inducing EMT signatures in prostate cancer cells and human tissues (Y. Sun et al., 2011). Without androgenic stimulation, AR expression is diminished during early ADT, but in the absence of AR, Zeb1 expression cannot be inhibited and thereby becomes increased. With increased Zeb1 transcription factor expression EMT promotion becomes transiently facilitated as a result of ADT leading to metastasis (Y. Sun et al., 2011). ZEB2 (SIP1) was originally described within the context of TGF-β signaling (Brabletz & Brabletz, 2010). ZEB2 interacts with SMADs and promotes tumorigenic invasion and down-regulates E-cadherin expression (Comijn et al., 2001). Expression of ZEB transcription factor has been correlated with progression to malignant carcinoma in various cancer types (including prostate), and expression could be induced by both estrogen and progesterone (Anose & Sanders, 2011; Chamberlain & Sanders, 1999). Identification of AREs in the promoter of the ZEB1 gene confirms that its expression is regulated by AR signaling (Anose & Sanders, 2011).

Identification of Enhancer of Zeste Homolog 2 (EZH2) from seminal studies by Chinnaiyan's group have enhanced our understanding regarding the role of epigenetic modifications in prostate, renal and breast cancer progression to EMT (Varambally et al., 2002). EZH2 expression is associated with cancer metastases and is localized in tumors with poor prognosis in combination with depressed E-cadherin, both markers associated with poor disease free survival (Bachmann et al., 2006; Laitenen et al., 2008). EZH2 functions as a histone lysine methyltransferase and its overexpression has been detected in mCRPC (Bachmann et al., 2006; M. M. Shen & Abate-Shen, 2010). Both EZH2 mRNA and protein levels are significantly elevated in prostate cancer compared to benign prostate hyperplasia (BPH) or human high grade PIN (HGPIN) (Li, Fan, & Fan, 2010); Functionally, EZH2 targets NKX3.1 inducing repression of the homeobox gene and this phenomenon is observed in up to 85% of HGPIN lesions and prostatic adenocarcinomas (M. M. Shen & Abate-Shen, 2010). Furthermore, EZH2 targets other genes undeniably linked to EMT, including E-cadherin and DAB2IP (Q. Cao et al., 2008; H. Chen, Tu, & Hsieh, 2005). The fusions of TMPRSS2, an androgen regulated gene, and the oncogenic ETS transcription factor ERG place ERG under androgenic drive (Hermans et al., 2006; Nam et al., 2007; Narod, Seth, & Nam, 2008; Perner et al., 2006; Rajput et al., 2007; Soller et al., 2006). ERG activates EZH2 transcription allowing the methyltransferase to induce its repressive epigenetic agenda (Yu, Yu, & Mani, 2010). The neuronal chemorepellant and tumor supressor gene SLIT2 has also been linked to EZH2 (Yu, Cao, et al., 2010). EZH2 targets SLIT2 and inhibits its expression under the drive of AR-dependent TMPRSS2-ERG fusion (Yu, Cao, et al., 2010). SLIT2 is down-regulated in a majority of prostate cancers and low levels of SLIT2 are associated with aggressive disease (Yu, Cao, et al., 2010). ERG overexpression interferes with AR binding to ARE/ARGs, thus providing an additional layer of selective pressure for AR overexpression and mutation, driving progression to CRPC (Yu, Yu, et al., 2010).

AR Cross-Talk with Growth Factor Signaling

The Wnt signaling pathway plays an important role in embryonic development and differentiation, and is a highly conserved pathway among organisms. The deregulation of Wnt signaling is associated with tumorigenesis and EMT (Yardy & Brewster, 2005). In prostate cancer cells, this pathway can engage in direct crosstalk with AR, with the central protagonist being β-catenin (Figure 1.2.B) (Yardy & Brewster, 2005). This molecule is located in distinct cellular locations: sequestered at the adherens junctions bonds to E-cadherin, in the cytoplasm, or in the nucleus (Yardy & Brewster, 2005). Wnt ligand binds with the seven pass transmembrane receptors: FZD (Frizzled) at the plasma membrane interface with the extracellular environment (Figure. 1.2.B). FZD receptors transduce a signal to Disheveled (Dvl) and Dvl subsequently dephosphorylates an associated protein Axin. Axin functions as a signaling scaffold protein coordinating the interactions of Adenomatous Polyposis Coli (APC), glycogen synthase kinase 3β (GSK3β), β-catenin, and Conductin (Figure. 1.2.B). The coordination of these proteins by Axin facilitates the phosphorylation of β-catenin and APC by GSK3β. The dephosphorylation of Axin diminishes its capacity to coordinate β-catenin in complex with GSK3β causing decreased phosphorylation of β-catenin (Figure. 1.2.B). The phosphorylation of β-catenin mediates subsequent ubiquitination and degradation, but without phosphorylation by GSK3β, β-catenin accumulates in the cytoplasm. Accumulation of β-catenin results in nuclear translocation of the protein and interaction with lymphoid enhancer binding factor 1/ T-cell factor (LEF1/TCF) transcription factors and transcription of β-catenin target genes, such as c-MYC, c-Jun and fra-1, in addition to EMT important urokinase type plasminogen activator receptor (uPAR), matrix metalloproteinase and cyclin D1 (Crawford et al., 1999; He et al., 1998; Marchenko et al., 2004; Tetsu & McCormick, 1999; Yardy & Brewster, 2005). AR and β-

catenin interact directly with one another (Figure. 1.2.B), impacting the EMT outcome (Robinson, Zylstra, & Williams, 2008). *In vitro,* androgen-stimulated transcriptional responses are enhanced by functional involvement of Wnt signaling, consequently opposing the effects of antagonistic anti-androgenic treatment (bicalutamide) (Truica, Byers, & Gelmann, 2000). This evidence implicates β-catenin as a co-activator of AR gene target transcription and potentially associated with emergence of CRPC (Yardy & Brewster, 2005). Furthermore, cognate ligand-induced AR signaling possesses the capacity to attenuate Wnt signaling and TCF/LEF1 dependent gene transcription.

The pioneering work of Arul Chinnaiyan's discovery of TMPRSS2: ERG gene fusion has been paramount in advancing our molecular understanding of prostate cancer pathophysiology (Clark et al., 2007; Tomlins et al., 2008). These gene fusions result in androgen driven expression of the transcription factor ERG. The consequences of these fusions on cell fate, are diverse and intriguing, but an important observation is that ERG fusion-positive tumors and Frizzled4 (Fzd4: 7 pass transmembrane receptor of Wnt signaling pathway) co-overexpression were consistently identified in clinical prostate cancer (Gupta et al., 2010). Moreover, overexpression of ERG induced the EMT phenomenon in androgen responsive cell lines (VCaP), including repression of E-cadherin and induction of N-cadherin. The effects of ERG overexpression could be abrogated by the modulation of FZD4, demonstrating that FZD4 was both necessary and sufficient to mediate the oncogenic effects of ERG overexpression and defining the impact of direct crosstalk of AR driven ERG overexpression with the Wnt signaling on prostate cancer EMT (Gupta et al., 2010).

TGF-β signaling is critical in diverse cell types by impacting important features of cellular behavior including migration, adhesion, alterations to the extracellular environment, apoptosis and promoting formation of osteoblastic metastatic lesions

(Derynck & Zhang, 2003; Siegel & Massague, 2003; M.-L. Zhu & Kyprianou, 2008). The TGF-β pathway traditionally engages signaling involving the SMAD proteins (Figure. 1.2.B) (Coffey Jr., Shipley, & Moses, 1986; Derynck & Zhang, 2003; Pu et al., 2009; Siegel & Massague, 2003; M.-L. Zhu, Partin, Bruckheimer, Strup, & Kyprianou, 2008). TGF-β signaling is mediated by the serine / threonine kinase domains of the TGFβRI and TGFβRII receptors and the formation of hetero-tetrameric complexes (Figure. 1.2.B) (Pu et al., 2009). Binding to TGF-β causes TβRII receptor to phosphorylate the regulatory GS domain of TβRI, initiating a downstream signaling cascade mediated by SMAD proteins (Beer et al., 2008; Derynck & Zhang, 2003). TβRI selectively phosphorylates regulatory SMADs (R-SMADs) at the SSXS motif on the carboxyl terminus of the SMAD (Derynck & Zhang, 2003; Siegel & Massague, 2003). The R-SMADs, SMAD2 and SMAD3, activated by the TβRI (Siegel & Massague, 2003), are sequestered in the cytoplasm via their interactions with SMAD anchor for receptor activation (SARA) (Siegel & Massague, 2003). Once activated by TβRI, R-SMADs lose affinity for SARA and become free to interact with SMAD4 (Siegel & Massague, 2003). SMAD4 is essential for formation of SMAD mediated transcriptional complexes, components of which are continuously shuttled between the cytoplasm and nucleus via nuclear pores (Derynck & Zhang, 2003; Siegel & Massague, 2003; B. Zhu & Kyprianou, 2005). The SMAD complex dictates transcriptional activation, via recruitment of co-activators such as p300, CBP, or SMIF. Conversely, for transcriptional repression, the SMAD complex recruits p107, SKI, SNON, TGIF, EVI1, and ZEB2 (SIP1) (Siegel & Massague, 2003; Ten Dijke, Goumans, Itoh, & Itoh, 2002). Expression of these co-regulators is dependent by cell type, developmental stage, and micro-environment hosted crosstalk facilitating a broad cellular response repertoire (Siegel & Massague, 2003).

EMT induction in response to TGFβ signaling engages oncogenic Ras or receptor tyrosine kinases (RTKs) to promote metastasis (Grunert, Jechlinger, & Meug, 2003; Huber et al., 2005). TGF-β is a ubiquitously expressed growth inhibitory cytokine (Siegel & Massague, 2003; B. Zhu & Kyprianou, 2005; M.-L. Zhu & Kyprianou, 2008; M.-L. Zhu et al., 2008). TGF-β contributes to tissue and organ homeostasis by inducing a system of proliferative vs apoptotic balances (Siegel & Massague, 2003; B. Zhu & Kyprianou, 2005). Defective / loss of TGF-β receptors and SMAD mutations are not directly responsible for the effects of EMT in cancer progression (M.-L. Zhu et al., 2008); rather, loss of apoptotic response occurs in cancer cells despite production of TGF-β ligand (Akhurst & Derynck, 2001).

Smad-independent signaling proceeds via MAPK pathways, involving activation of Erk, JNK, and p38 MAPK signaling pathways by TGF-β. Oncogenic Ras contributes to the activation of Erk/MAPK signaling, in a context dependent manner. TGF-β activates TGF-β activated Kinase 1 (TAK1), a MAPK kinase kinase family member (MAPKKK), leading to activation of JNK and p38 MAPK. TAK1 can also phosphorylate IκB, thereby activating NFκB signaling (Derynck & Zhang, 2003). A direct mechanistic link of EMT to cancer progression is mediated by the effect of TGF-β signaling on activation of Rho A (Figure. 1.2.B). Rho A and p160ROCK (effector kinase) activation in conjunction with activation of Cdc24, p38, MAPK, and Smad signaling, correlate with stress fiber formation, membrane ruffling, lamellipodia formation and the physical mechanisms of EMT (Derynck & Zhang, 2003). Rho A is upregulated in prostate cancer cells as compared to the benign prostate and this elevated expression is linked to aggressive disease and diminished disease free survival in patients after radical prostatectomy (Schmidt et al., 2012). In fact, Rho A activation by TGF-β is similarly activated by action of AR on Serum Response Factor target genes further corroborating the cross talk

between TGF-β and AR (Schmidt et al., 2012), in the context of EMT cellular "landscaping".

Significantly, elevated TGF-β correlates with increasing tumor grade in numerous human malignancies, including prostate cancer (Bierie & Moses, 2006; Ivanonvic, Melman, Davis-Joseph, Valcic, & Gelieber, 1995; Levy & Hill, 2006; Wojtowicz-Praga, 2003). And furthermore, overexpression of TGF-β ligand is detected in advanced prostate cancer (Coffey Jr. et al., 1986; Derynck & Zhang, 2003; B. Zhu & Kyprianou, 2005). TGF-β ligand binds to and induces phosphorylation of TβRI by TβRII resulting in SMAD signaling in prostate cancer cells. SMADs 3 and 4 serve as transcriptional co-regulators of AR target genes and conversely, ligand bound AR transcriptionally modulates SMAD3 in prostate cancer (Song et al., 2010; van der Poel, 2005). SMAD4 (alone or in conjunction with SMAD3) can co-regulate AR transactivation via binding to the DBD and LBD domains of the steroid receptor thereby modulating its DHT induced activity (M.-L. Zhu & Kyprianou, 2008; M.-L. Zhu et al., 2008). SMAD3 can bind AR as well, but this interaction is mediated by the NTD (Hayes et al., 2001). In a mechanistic twist, AR overexpression enables prostate cancer cells to overcome the growth inhibitory effects of TGF-β under DHT deprived conditions (van der Poel, 2005). Moreover, expression of SMAD3 enhances AR mediated transactivation, whilst co- overexpression of SMAD3 and 4 repressed AR transactivation (Kang et al., 2002). The TGF-β/Smad signaling pathway elicits a downstream activation in Snail thereby repressing E-cadherin expression in a number of cancer cell types (Thuault, Peinado, Cano, Heldin, & Moustakas, 2008). In LNCaP TβRII human prostate cancer cells, DHT (alone or in combination with TGF-β) significantly induced Snail expression (M.-L. Zhu et al., 2008), pointing to a dynamic crosstalk between the AR and TGF-β pathways in control of EMT. Recent studies identified a role for Hexim-1 in mediating such a cross-talk between AR

and TGF-β in prostate cancer progression. Hexim-1 is an inhibitor of cyclin dependent kinase 9 (Cdk9) of transcription elongation factor (pTEFb) complex, which is upregulated and translocated to the cytoplasm during tumor progression (Mascareno, Belashov, Siddiqui, Liu, & Dhar-Mascareno, 2012). Cdk9 interacts with AR and phosphorylates the AR at serine 81 (Lee & Chang, 2003), and transcriptionally programs Smads 1 and 3 via phosphorylation of linker region (Alcaron et al., 2009; Mascareno et al., 2012). Such refined mechanistic control of Hexim-1 expression, supports its role as a converging modifier of activity for AR and TGF-β signaling cross-talk towards EMT (Mascareno et al., 2012).

Notch signaling is fundamentally significant in development and tissue homeostasis. Notch signaling facilitates an important mode of cell-cell communication. Notch proteins (1-4) are type I , single pass transmembrane receptors (Bolos, Grego-Bessa, & de la Pompa, 2007). The extracellular domain of the Notch protein participates in ligand binding and is composed of a variable number of epidermal growth factor (EGF) like domains (essential for ligand binding) and three cysteine rich LIN12/Notch repeats (LNR) (ensure signaling only transduced in presence of ligand)(Bolos et al., 2007; Rebay et al., 1991). The intracellular domains of the Notch receptor include RAM23 domain, six ankyrin / cdc10 repeats, two nuclear localization signals, transcriptional activation domain and a PEST sequence (Bolos et al., 2007). The ligands recognized by the Notch receptor are Delta 1,3, and 4 as well as Jagged 1 and 2; these ligands are membrane bound and composed of an amino terminal domain known as DSL and variable number of EGF like repeats (Bettenhausen, Hrabe de Angelis, Simon, Guenet, & Gosler, 1995; Dunwoodie, Henrique, Harrison, & Beddington, 1997; Lindsell, Shawber, Boulter, & Weinmaster, 1995; Shutter et al., 2000). The Jagged ligands possess a cysteine rich (CR) domain and ligand-receptor initiated signaling cascade

results in cleavage of the Notch receptor and ultimately translocation of the Notch intracellular domain (NICD) to the nucleus. As discussed above, Snail1 is a transcription factor responsible for repressing E-cadherin transcription, with Notch1 activation upstream of Snail1 (Bolos et al., 2007). This observation has been further validated in the immortalized porcine aortic endothelial cell line, whereby overexpression of NICD induced an EMT via activation of Snail1 and subsequent repression of E-cadherin (Timmerman et al., 2004). The correlation between expression of Notch ligand, Jagged1, and high grade and metastatic prostate cancer compared to localized prostate cancer (Santagata et al., 2004) is of major translational value as Jagged1 may serve as an independent prognostic indicator of prostate cancer recurrence and progression, potentially driven by a link with androgenic signaling (Bailey, Singh, & Hollingsworth, 2007; Santagata et al., 2004). Notch1 signaling is associated with osteoblast differentiation and Notch1 expression is markedly elevated in osteoblast skeletal derived prostate cancer cells (Bailey et al., 2007), evidence indirectly supporting its involvement in EMT driven prostate tumor metastasis to the bone.

Androgen Deprivation Therapy

CRPC is a disease addicted to AR signaling developed through the course of androgen deprivation therapy (ADT). ADT is effective in patients by inducing tumor regression via tumor apoptosis (Kahn, Collazo, & Kyprianou, 2014). ADT induces a chemical castration state in the patient characterized by serum testosterone levels <50ng/mL (Kahn et al., 2014). AR signaling can become reactivated through a variety of mechanisms which have been reviewed elsewhere (B. J. Feldman & D. Feldman, 2001). Several mechanisms can be engaged by prostate tumors to bypass or perpetuate AR

signaling toward CRPC (Brinkmann et al., 1999; B. J. Feldman & D. Feldman, 2001; Knudsen & Penning, 2010). These mechanisms lend credence to the need for personalized medicine and continued research in the landscape of alternate pathways to CRPC (Brinkmann et al., 1999; B.J. Feldman & D. Feldman, 2001; Knudsen & Penning, 2010). Alterations to the regulation, structure and post-translational modifications of the AR itself can perpetuate continued androgen signaling, and AR has been shown to be commonly overexpressed in CRPC (C. D. Chen et al., 2004). However, one must consider the structural changes in AR as functional contributors to therapeutic resistance. Point mutations increasing the affinity of the AR for ligand have been identified causing the pathway to become hypersensitive (Gregory, Johnson, Mohler, French, & Wilson, 2001). Promiscuous mutations cause binding flexibility in the LBD allowing the AR to become activated by adrenal androgens, androgenic metabolites, and even some anti-androgen therapeutics such as flutamide and bicalutamide (B. J. Feldman & D. Feldman, 2001; Marcelli et al., 2000; Taplin et al., 2001; Tilley, Buchanan, Hickey, & Bentel, 1996). Over twenty splice variants of AR, some lacking LBD, and therefore constitutively active have been identified and associated with progression of CRPC and metastasis (Dehm, Schmidt, Heemers, Vessella, & Tindall, 2008; Z. Guo et al., 2009; Hu et al., 2009; Hu, Isaacs, & Luo, 2010; Jenster et al., 1995; S. Sun et al., 2010). AR can be activated independent of ligand interactions by aberrant signaling pathways causing activation of the protein and homo-dimerization by growth factors, receptor tyrosine kinases and the Akt pathway via loss of PTEN (Craft, Shostak, Carey, & Sawyers, 1999; Culig et al., 1994; B. J. Feldman & D. Feldman, 2001; J. H. Li et al., 1997). Growing evidence by a number of investigative teams support the abilities for prostate cancer cells to synthesize their own androgens “hijacking” adrenal synthesis enzymes (Knudsen & Penning, 2010; Locke et al., 2008; Montgomery et al., 2008; Stanbrough et al., 2006). The entire AR signaling axis can even be bypassed by

overexpression of the apoptosis blocking protein, Bcl2, which is frequently found overexpressed in prostatic intraepithelial neoplasia (PIN).

Abiraterone**:** Abiraterone Acetate (AA) is a novel anti-androgen therapy designed to target the adrenal androgen mediated signaling axis by blocking the synthesis of adrenal products which serve as precursors for testosterone and DHT synthesis (Figure 1.3) (Di Lorenzo, Buonerba, De Placido, & Sternberg, 2010; A. O. Sartor, 2011; Walcak & Carducci, 2007). AA acts as a pregnenolone analog, inhibiting the rate limiting enzyme, cytochrome P450 (CYP17A1), further inhibiting androgen biosynthesis (Di Lorenzo et al., 2010; A. O. Sartor, 2011). AA inhibits both the 17α-hydroxylase and 17,20 functions of CYP17A1 (Di Lorenzo et al., 2010). The efficacy of AA was demonstrated in the COU-AA-301 trial, confirming that AA imparted additional survival benefit compared to DR-CRPC men treated with placebo and prednisone. In addition to overall survival increase, benefits were seen with regard to time to disease progression, biochemical recurrence and tumor burden (De Bono, Logothetis, et al., 2010; Di Lorenzo et al., 2010; A. O. Sartor, 2011).

Enzalutamide (MDV3100)**:** The importance of AR targeting in DR-CRPC is highlighted by the development of the direct, AR antagonist, MDV3100 (Di Lorenzo et al., 2010; H. C. Shen & Balk, 2009). This drug is a diarylthiohydantoin member of the family of AR antagonists rationally designed from the crystal structure of the AR bound to its ligand (H. C. Shen & Balk, 2009). MDV has been shown to be effective in the context of AR overexpression, in addition to inhibiting AR nuclear translocation, preventing binding of the AR to DNA, blocking recruitment of co-activators to AR target genes, and induces apoptosis (Figure 1.3) (Scher et al., 2010; H. C. Shen & Balk, 2009; Vishnu & Tan, 2010). MDV has been shown to be efficacious in improving survival in therapy naïve patients, those previously treated with ADT, and in patients previously

treated with Docetaxel and in patients treated with both (Attard, Cooper, & de Bono, 2009; Di Lorenzo et al., 2010; Nadal et al., 2014; Tran et al., 2009b). MDV inhibits translocation of full length AR and prevents activation of AR splice variants (ARv) lacking portions or all of the LBD by preventing dimerization of those ARv with full length AR (Watson et al., 2010). Interestingly, MDV treatment and other ADT strategies has been shown to induce expression of ARVs, which emphasizes the need to identify effective therapeutic targets to impair CRPC outside of the "classic" AR signaling (Watson et al., 2010).

N terminal domain targeting EPI-002: Anti-androgenic targeting of AR has primarily been focused on binding LBD and thereby preventing activation of AR, but one AR antagonist has emerged which takes an enlightened approach. The small molecule inhibitor EPI-001/002 series of anti-androgens bind the intrinsically disordered domain of the NTD preventing it from initiating transcription activation functions and DNA binding (Figure 1.3) (Andersen et al., 2010). The EPI series was originally identified from peptides isolated from marine sponges (Sadar, 2011). This novel anti-androgen has demonstrated efficacy in androgen-dependent and CRPC models of prostate cancer s and garnered much attention (Andersen et al., 2010). EPI small molecule inhibitors offer a unique feature to the AR targeting game in that they can bind and inhibit many of the AR variant isoforms expressed in advanced prostate cancer tumors (Martin, Banuelos, Sadar, & Kyprianou, 2014; Sadar, 2011). Characteristic of AR variant structure, the C-terminus, exons within, or the entire LBD is missing. This would prevent activity with most traditional anti-androgens, activity of EPI is not deterred. EPI can still effectively bind and inhibit activation of many AR variant isoforms as data generated in this thesis demonstrate (Martin et al., 2014).

Classical Taxane Action

Taxanes are derived from naturally occurring molecules identified in the bark of yew trees (Huizing et al., 1995). Historically, the anti-tumor action of taxanes has been attributed to their inherent ability to bind and stabilize the architectural component of the cell: microtubules (Huizing et al., 1995). The classic understanding of the mechanism of anti-tumor action of taxanes is that they bind and stabilize the interaction between two sub-units of β tubulin, preventing de-polymerization of the protofilament sub-structure within the microtubule (Figure 1.3 & Figure 1.4) (Kraus et al., 2003). This stabilization results in G2M arrest and apoptosis (Huizing et al., 1995; Kraus et al., 2003). Bcl-2 overexpression is frequently observed in prostate cancer, and taxanes are capable of counteracting the effects of this pro-survival protein of the apoptotic pathway (Bruckheimer & Kyprianou, 2001, 2002; Debes & Tindall, 2004b; Oliver et al., 2005).

The clinical evidence for the use of taxanes in patients with CRPC emerged from a series of clinical trials, TAX327 and SWOG (Southwest Oncology Group) 9916, which demonstrated a significant survival benefit of Docetaxel based treatment compared to control treated patients (Tannock et al., 2004). Docetaxel treatment conferred palliative relief and overall survival benefits (Berthold et al., 2008; Petrylak et al., 2004). Since FDA approval, taxane chemotherapy stood alone for nearly a decade as the only clinically relevant intervention for mCRPC patients. Unfortunately, taxane treatment ultimately fails and, the majority of patients develop resistance. The molecular mechanisms driving therapeutic resistance to microtubule targeting chemotherapy in prostate cancer after Docetaxel treatment are not fully understood. Some of the mechanisms of resistance can be attributed to the adenosine triphosphate dependent drug efflux pump P-glycoprotein-1. Docetaxel has a high affinity for this pump and an increase in expression of the efflux pump itself is observed over the course of prostate

cancer progression (Abdulla & Kapoor, 2011; Attard, Greystroke, Kaye, & De Bono, 2006). Biochemical recurrence is often associated with other clinical manifestations; bone, brain, and lymph node metastasis as well as increasing amounts of pain secondary to the metastatic lesions are found in CRPC patients (Abdulla & Kapoor, 2011).

Cabazitaxel**:** Cabazitaxel is a novel, next-generation semi-synthetic Taxane chemotherapeutic drug that has been shown to be effective in the Docetaxel resistant-CRPC landscape (Figure 1.3) (Galsky, Dritselis, Kirkpatrick, & Oh, 2010; A. O. Sartor, 2011). Cabazitaxel is structurally very similar to Docetaxel save that in place of hydroxyl groups there are methoxyl groups in those positions (Azarenko, Smiyun, Mah, Wilson, & Jordan, 2014; Vrignaud et al., 2013). Cabazitaxel is highly cytotoxic and has a low affinity for the adenosine triphosphate-dependent drug efflux pump: P-glycoprotein 1, known to confer chemotherapeutic resistance (Di Lorenzo et al., 2010). Unlike Docetaxel, Cabazitaxel can cross the blood brain barrier and has a 95 hour terminal half-life in humans (versus 12 hours for Docetaxel) (Bruno & Sanderink, 1993; Cisternino, Bourasset, & Archimbaud, 2003; Sanofi-Aventis, 2014; Schutz, Buzaid, & Sartor, 2014). Cabazitaxel was shown in a multicenter, randomized; phase 3 clinical trial (Treatment of Hormone-Refractory Metastatic Prostate Cancer (TROPIC)) to impart a statistically significant increase in overall survival (De Bono, Oudard, Ozguroglu, Hansen, Machiels, Kocak, Gravis, Bodrogi, Mackenzie, Shen, Roessner, Gupta, & Sartor, 2010; Galsky et al., 2010). Tumor response, biochemical recurrence, and tumor progression all favored the Cabazitaxel treatment and was approved by the US Food and Drug Administration for use in DR-CRPC patients (De Bono, Oudard, Ozguroglu, Hansen, Machiels, Kocak, Gravis, Bodrogi, Mackenzie, Shen, Roessner, Gupta, & Sartor, 2010; Galsky et al., 2010; A. O. Sartor, 2011). In addition to imparting overall survival benefits to

chemotherapeutic naïve patients, the exciting findings associated with Cabazitaxel are its ability to confer additional overall survival benefits in patients who have already experienced biochemical recurrence on ADT, Docetaxel chemotherapy, or both (Abdulla & Kapoor, 2011; A. O. Sartor, 2011).

Beyond Microtubule Stabilization

In addition to the intramolecular ballet required for the AR to induce conformational change suitable to facilitate nuclear translocation, AR must be physically transported from the cytoplasm to the nucleus; a feat accomplished via motorized translocation along microtubules (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011). Work from our lab revealed, for the first time, that in addition to stabilizing microtubules and inducing G2M arrest, taxanes are particularly poignant in prostate cancer, because they possess the ability to deter translocation of the AR to the nucleus and inhibit AR driven gene transcription (Figure 1.4) (M.-L. Zhu et al., 2010b). Using clinical specimens from patients treated with Docetaxel versus untreated, immunohistochemical analysis of tissue microarrays strikingly revealed significantly diminished AR nuclear localization in the Docetaxel-treated patients (M.-L. Zhu et al., 2010b). Although, AR protein expression was not affected by the treatment, nuclear localization was diminished by 38%, thereby highlighting the mechanistic effect of Docetaxel chemotherapeutic treatment. Further investigation into the domain of the AR responsible for mediating the interaction with the taxane target tubulin, revealed that the NTD negotiated this association (M.-L. Zhu et al., 2010b). These data were confirmed and extended by the finding that AR nuclear translocation is inhibited in circulating tumor cells from patients treated with Docetaxel (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011). These important mechanistic insights serve as a roadmap to

understanding why taxane chemotherapeutics served as our only clinically relevant treatment for CRPC for nearly a decade and guide our pursuit for future therapeutics.

Forkhead box protein 1 (FOXO1) has been shown to inhibit AR activity by binding and sequestering AR in the nucleus, and not allowing it to bind target AREs (Figure 1.4) (P. Liu, Li, Gan, Kao, & Huang, 2008). Taxane treatment can cause FOXO1 nuclear translocation such that any AR reaching the nucleus may be bound by FOXO1 and thus unable to initiate target gene activation (Gan et al., 2009b; P. Liu et al., 2008).

Microtubules do not represent a transportation mechanism. Microtubules are merely a highway *along* which cargo is transported by ATP-dependent motor proteins: dynein and kinesin. Dynein motor proteins transport cargo in a “minus” end direction along microtubules from the cytoplasm to the nucleus. Dynein motor trafficking is important to efficiently transport AR from the cytoplasm to the nucleus as demonstrated by co-immunoprecipitation of full length AR with dynein in prostate cancer cells (Figure 1.4) (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011). Furthermore, this association is increased following androgen ligand stimulation. In cells expressing full length AR, overexpression of dynein linker protein “dynamitin” prevented translocation of AR to the nucleus upon androgen stimulation because cargo proteins could not be loaded onto motor proteins (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011). AR splice variants are expressed with high prevalence in CRPC and their presence has been implicated in disease progression (Sprenger & Plymate, 2014). Recent work has demonstrated that AR variant identity determines it’s ability to interact with and be transported by dynein (Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, S.R., et al., 2014). ARv567es (an AR protein which has both N and C termini but lacks exons 5-7) associates with and is translocated by dynein, whereas ARv7 (lacks complete ligand binding domain) does not interact

directly with dynein or microtubules and localizes to the nucleus (Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, S.R., et al., 2014).

Kinesins have been implicated as contributors to cancer progression, and their role has been eloquently reviewed elsewhere (Rath & Kozielski, 2012). More than 45 kinesins are encoded in the human genome with 14 subfamilies based on phylogenetic analysis of the motor domain. As the counterparts to dynein, kinesins move in a "plus" end direction along microtubules from the nucleus to the cytoplasm and perform two essential functions: facilitate progression of different stages of cell division and are important for intracellular vesicle and organelle transport. As schematically illustrated in Figure 1.4, kinesin motor proteins consist of a long coiled-coil stalk with a cargo binding module at one end and globular motor domain (head) at the other (Wiltshire et al., 2010).

Eg5 is a kinesin involved in bipolar spindle formation during mitosis. It forms homo-tetramers of two antiparallel dimers with motor domains on both ends; this allows Eg5 to grasp two microtubules and utilize its' plus end directed motor activity to push them apart (Rath & Kozielski, 2012). Inhibition of Eg5 activates the spindle check point induces mitotic arrest and apoptosis, and therefore it is an attractive target for therapeutic targeting. Several Phase I and Phase II clinical trials have worked to bring Eg5 inhibitors to patients. One such inhibitor, S-Trityl-L-Cysteine (STLC) showed efficacy in Docetaxel-resistant prostate cancer cells and STLC was not affected by P-Glycoprotein upregulation (Wiltshire et al., 2010). Of clinical significance is a recent retrospective study that identified Eg5 nuclear expression as a predictive biomarker of Docetaxel response in mCRPC patients and a prognostic biomarker for hormone naïve prostate cancer patients (Wissing et al., 2014).

Mitotic centromere-associated kinesin (MCAK) is a member of the kinesin-13 subfamily and is a non-motile, microtubule depolymerizing kinesin that targets to microtubule tips and utilizes its' ATP hydrolysis power stroke to "flick" tubulin subunits off the end of the structure during mitotic progression (Desai, Verma, Mitchison, & Walczak, 1999; Ogawa, Nitta, Okada, & Hirokawa, 2004; Rath & Kozielski, 2012). The mitotic kinase Aurora B controls that localization and activity of MCAK at the centromere/kinetochore while Aurora A controls the same functions at the spindle poles (Sanhaji et al., 2010; Sanhaji, Friel, Worderman, Louwen, & Yuan, 2011; X. Zhang, Lan, Ems-McClung, Stukenberg, & Walczak, 2007). Polo-like kinase 1 (Plk1) regulates MCAK enzymatic activity and actually controls microtubule de-polymerization in cells making it of particular interest in taxane resistant cells (Sanhaji et al., 2011). In normal cells, MCAK tracks to the plus end tips of microtubules and utilizes microtubule depolymerizing properties to correct improper kinetochore attachments at the centromere during mitosis. During interphase, MCAK localizes to the plus end of microtubules and actively depolymerizes them (Sanhaji et al., 2011). a variety of cancer types, MCAK becomes grossly overexpressed. Indeed in diverse human tumors, data mining has revealed MCAK as an important protein overexpressed in CRPC data sets and is indicative of taxane chemotherapy resistance (Sircar et al., 2012). Early attempts to target MCAK via inhibitors have been described (Aoki, Ohta, Yamazaki, Sugawara, & Sakaguchi, 2005; Rickert, 2008). My recent studies show that while MCAK expression may be indicative of Docetaxel resistance, MCAK expression may actually be a target of Cabazitaxel (Figure 1.4). Furthermore, in castration resistant 22Rv1 and androgen dependent VCaP, and LNCaP cell lines MCAK expression was decreased over time in both the presence and absence of androgens (Figure 1.4).

HSET is a member of the Kinesin-14 subfamily and like the rest of this subfamily is a “minus” end directed motor protein unlike the rest of the kinesins. HSET promotes proper bi-spindle pole formation and facilitates proper cytokinesis. Furthermore, HSET has been shown to be overexpressed in Docetaxel resistant tumors (De, Cipriano, Jackson, & Stark, 2009). Chromosomal instability is common in tumor cells leading to missegregation, aneuploidy and cell death therefore, undergoing mitosis represents an additional challenge for cancer cells (Kwon et al., 2008). Centrosomal amplification and subsequent clustering has evolved to prevent tumor cells with aneuploidy chromosomes from undergoing apoptosis (Chandhok & Pellman, 2009). HSET has emerged as an essential regulator of centrosomal clustering and an attractive therapeutic target. Normal mitotic cells possess exactly two centrosomes, and thus do not require HSET to coordinate the clustering of their centrosomes, whilst tumor cells who cannot appropriately cluster their centrosomes undergo mitotic arrest and apoptosis (Chandhok & Pellman, 2009). HSET remains elusive as a druggable target, but recent evidence from our lab has shown that HSET expression is down regulated at both the mRNA and protein level by Cabazitaxel treatment (Martin et. al, 2015).

Mechanisms of Therapeutic Resistance

ATP binding cassette (ABC transporter) P-glycoprotein (P-gp) / Multidrug resistance protein (MDR) is overexpressed in the cell membranes of tumors, and its overexpression also has been identified in clinical specimens from CRPC patients (Figure 1.5.A) (Mahon, Henshall, Sutherland, & Horvath, 2011; Siegsmund, Kreukler, & Steidler, 1997; Theyer, Schirmbock, & Thalhammer, 1993). This protein serves as a membrane bound efflux pump physically pumping a wide range of substrates, including

Docetaxel, out of treated tumor cells compromising the effect on stabilizing microtubules. Variable response to P-gp can be seen in chemoresistant prostate cancer cell lines. Chemoresistant PC3 cells do not overexpress P-gp, whilst chemoresistant DU145 do overexpress P-gp and that resistance can be modulated with knockdown thereof (Mahon et al., 2011; Makarovskiy, Siryaporn, Hixson, & Akerley, 2002; Takeda et al., 2007). Another member of the MDRP family, lesser known MRP1 is up-regulated in chemoresistant prostate cancer cell lines which do not over-express P-gp (Zalcberg et al., 2000). Together the expression of ABC transporters and subsequent effects on chemo-resistance appear to be modulated by p53 and PIM1 kinase (Mahon et al., 2011; Sullivan et al., 2000; Xie et al., 2008). Hydroxyl groups on the structure of Docetaxel are substituted for methoxyl groups in the structure of Cabazitaxel (Vrignaud et al., 2013). Interestingly, in MCF7 breast cancer cells, Cabazitaxel was taken up faster than Docetaxel and washing did not affect intracellular concentration of the drug (unlike Docetaxel treated MCF7 cells which demonstrated 50% concentration of drug post washing). These data indicate that Cabazitaxel is better retained by cancer cells (Azarenko et al., 2014). Despite this improvement in intracellular retention, MDR resistant MCF-7 variant cells still demonstrated resistance to the effects of Cabazitaxel but it showed much less cross resistance than paclitaxel and Docetaxel (Duran et al., 2014).

Modulation of the actual microtubule and their component tubulin is another important mechanism of resistance to taxanes. Mutation of the taxane binding site of β-tubulin or isotype switching may confer resistance to taxane action and also may modulate binding of microtubule associated proteins (MAPs) (Figure 1.5.B) (Giannakakou et al., 2000; Huzil, Chen, Kurgan, & Tuszynski, 2007; Madan, Pal, Sartor, & Dahut, 2011). There are at least seven isotypes of β-tubulin known and the

predominant isotype expressed in normal tissue is isotype I (Luadena, 1998; Mahon et al., 2011). Increased expression of isotype β III tubulin has been clinically demonstrated for lung, breast and ovarian cancers; in addition, overexpression of the β III tubulin isoform has been associated with progression to mCRPC is predictive of Docetaxel efficacy, and may further serve as a *theranostic* biomarker (Ploussard et al., 2010; Terry et al., 2009). Cabazitaxel is also subject to therapeutic resistance induced by expression of β III tubulin isotype (Duran et al., 2014).

Furthermore, alterations of microtubule binding dynamics also contribute to taxane resistance. The binding site of taxane only exists on microtubules when tubulin is polymerized and not on tubulin dimers (Orr, Verdier-Pinard, McDaid, & Band Horwitz, 2003). Therefore, a shift toward preference of the dimerized form of tubulin and away from polymerized tubulin would represent a survival advantage for cells exposed to taxanes (Figure 1.5.C). Certain taxane resistant cell lines have developed a requirement for exposure to taxane suggesting that the microtubules have become “hypostable” and must be exposed to taxane to stabilize their microtubules adequately for normal function (Orr et al., 2003). For example, A549 cells continually exposed to taxane were selected for resistance and were developed to be between nine and 17 fold more resistant than parental cells. These cells were dependent on exposure to taxanes for growth, were blocked at G2/M phase of the cell cycle in its absence, and exhibited increased dynamic instability towards dimerized tubulin (Goncalves et al., 2001; Orr et al., 2003). Recently, ERG was implicated in affecting the shift of microtubules toward instability (Galletti et al., 2014). ERG rearrangements are the most frequently recurring genetic alteration in human prostate tumors and are known to undergo gene fusion with the 5’ promoters of TMPRSS2, SLC45A3 and NDRG1 (Tomlins et al., 2005). In addition to playing a causative role in transformation of normal prostate epithelium, ERG has now been

shown to bind soluble tubulin in the cytoplasm and shift tubulin binding dynamics toward catastrophe contributing to taxane resistance (Galletti et al., 2014).

Earlier studies by Giannakakou et al. identified two taxane resistant human ovarian carcinoma cell lines that are 24 fold more resistant to taxanes but hypersensitive to the microtubule depolymerizing agent Vinblastine (Giannakakou et al., 1997; Orr et al., 2003). The primary β-tubulin isotype βI was identified to have a Phe_{270} to valine substitution in one cell line and an Ala_{364} to threonine substitution in the other (Giannakakou et al., 1997). These substitutions exist close to the region of the tubulin domain responsible for interacting with the taxane ring system, and are thus are capable of disrupting taxane binding (Orr et al., 2003).

Altered expression of Microtubule Associated Proteins (MAPs) is associated with progression to chemotherapeutic resistance in prostate cancer (Figure 1.5.E). Stathmin is a microtubule-destabilizing protein that plays an important role in mitotic spindle formation and disassembly (Belmont & Mitchison, 1996; Marklund, Larsson, Gradin, Brattsand, & Gullberg, 1996; Mistry & Atweh, 2006). Stathmin is expressed at very high levels in a variety of human cancers including prostate cancer and its expression has been shown to correlate with malignant phenotype of the cancer. Stathmin may serve as a prognostic marker of progression in prostate cancer (Friedrich, Grongberg, Landstrom, Gullberg, & Bergh, 1995). Inhibition of stathmin expression has been associated with shift towards EMT in prostate cancer and the extent of its expression is stage specific (Williams et al., 2012). Overexpression of stathmin may contribute to taxane resistance by shifting the equilibrium between soluble and polymerized tubulin dimers towards un-polymerized tubulin counteracting the action of tubulin polymer stabilizing taxanes. Treatment of prostate cancer cells (LNCaP) with stathmin interference plus taxane induces a synergistic effect on decreasing viability compared to either treatment alone

(Mistry & Atweh, 2006). Conversely, MAP4 is a microtubule stabilizing protein and phosphorylation of MAP4 causes it to dissociate from microtubules resulting in the loss of stabilizing function (Chang et al., 2001; Chien & Moasser, 2008). Increased phosphorylation of MAP4 has been associated with a decrease in taxane sensitivity in ovarian cancer cell lines (Poruchynsky et al., 2001). Furthermore, MAP4 expression is suppressed in the presence of wild-type p53 (Murphy, Hinman, & Levine, 1996; C. C. Zhang et al., 1998).

Among MAPs whose activity becomes altered through the course of development of taxane resistance, ATP-driven motor protein kinesins have emerged as key players (Figure 1.5.F). In breast cancer, KIFC3, KIFC1, and KIF5A can confer or enhance docetaxel resistance (X. Liu, Gong, & Huang, 2013). In prostate cancer and others, KIF2A (MCAK) overexpression has also been shown to confer or enhance Docetaxel resistance (Ganguly, Yang, & Cabral, 2011; Sircar et al., 2012). Others: KIF2B, CENPE, and HSET have also been implicated in resistance to microtubule targeting (Schmizzi, Currie, & Rogers, 2010; Yang, Liu, Ikui, & Horwitz, 2010). In all cases, the ATP-binding domain of the kinesin is required to enhance taxane resistance (Tan et al., 2012). With regard to MCAK, this kinesin is a plus end tracking, microtubule depolymerizing kinesin, which induces an unnatural bend conformation to the microtubule structure inducing the removal of tubulin subunits from the end of the structure. These kinesins therefore work to counteract the effect of taxanes by depolymerizing the ends of microtubules which have been stabilized. Overexpression of these kinesins allows tumor cells to “out run” the effects of taxanes (De et al., 2009; X. Liu et al., 2013; Tan et al., 2012).

EMT as a mechanism of resistance: Recent evidence has implicated that resistance to Cabazitaxel chemotherapy can be induced by EMT (Figure 1.5.G) (Duran

et al., 2014). The significance of EMT in cancer emerges as tumor cells must physically detach from their immediate primary tumor, invade into the surrounding microenvironment, intravasate into the vasculature, endure the turbulence of circulation in the blood stream or lymphatics, and extravasate from the circulatory system at a secondary site (Kalluri & Weinberg, 2009; Yilmaz & Christofori, 2009). Each step required for execution of EMT requires a vast number of molecular events (Kalluri & Weinberg, 2009; Matuszak & Kyprianou, 2011a; Yilmaz & Christofori, 2009). Epithelial cells must begin their transition to a mesenchymal phenotype by disrupting their intercellular adhesive contacts (Acloque et al., 2009). This initial modification occurs by formation of apical constrictions and disorganization of the basal cytoskeleton resulting in detachment and loss of apical-basal organization (Acloque et al., 2009; Barrallo-Gimeno & Nieto, 2005; Moreno-Bueno, Portillo, & Cano, 2008; Peindao, Olmeda, & Cano, 2007). The phenotype of the detached cell becomes spindle-like and exhibits a front-rear polarity conferring enhanced motility and invasive shape (Kalluri, 2009; Matuszak & Kyprianou, 2011a; Thiery et al., 2009; J. Yang & R. Weinberg, 2008). Furthermore, breakdown of the basal membrane and extracellular matrix must occur for migration to ensue and this is accomplished via secretion of proteases and acquisition of migratory/invasive properties (Acloque et al., 2009; Haraguchi et al., 2008). Using gene expression profiling techniques, Cabazitaxel resistant tumor cells were shown to possess alterations in the expression of EMT marker profiles. Specifically, they exhibited increased expression of the mesenchymal marker, vimentin, and decreased expression of the epithelial marker, E-cadherin, compared to parental controls (Duran et al., 2014).

Overcoming Taxane Resistance in Clinical Setting: Intermittent Chemotherapy. Administering cytotoxic taxane chemotherapies is associated with significant toxicity in patients. One tactic that has been successfully employed is the use of intermittent

chemotherapy. Patients enjoy “drug holidays” or breaks in therapy during which they may be able to recover from cumulative toxicity of prolonged treatment (Madan et al., 2011). In fact, allowing time for patients to resolve drug side effects may allow taxane therapy to be extended longer than otherwise would be tolerated by the patient (Beer et al., 2008; Bellmunt, Albiol, & Albanell, 2007). Furthermore, it is attractive to hypothesize that intermittent chemotherapy prevents selection for “taxane-resistant” cell population and potentially circumvents development of resistance (Madan et al., 2011). This intermittent approach was directly evaluated in the ASCENT trial in which 250 patients were administered 36mg/m^2 Docetaxel weekly with high dose calcitriol or placebo. The parameters of the study established guidelines regarding eligibility for drug holiday. In order to qualify for such a treatment break, patients must exhibit a PSA decline of >50% to a level less than 2.0 ng/mL. After Docetaxel intervention, 45 of the 250 enrolled patients (18%) were eligible for intermittent chemotherapy based on the rigorous parameters described. The treatment holidays lasted until there was evidence of disease progression or increase of PSA by 50% to a value of greater than 2.0 ng/mL. The median holiday lasted greater than or equal to 20 weeks for these patients. Of those patients given treatment holiday, 90.9% responded to treatment after resumption of chemotherapy. Responsiveness to chemotherapy was measured as either a second decline in PSA of 50% or stabilization of PSA level (stop increasing PSA) (Beer et al., 2008). Since then, further investigation has lent credence to the concept of intermittent chemotherapy (Kelly et al., 2012; Lin, Ryan, & Small, 2007; Ning et al., 2010). Specifically, a phase II study from the National Cancer Institute investigating the combination of Docetaxel with anti-angiogenesis therapeutics reported a median overall survival of more than 28 months with employment of intermittent Docetaxel administration, representing a huge survival advantage (Ning et al., 2010).

Combination Strategies

Platinum Based Therapy. An attractive combination therapy is combining the microtubule stabilizing action of taxanes with the cytotoxic, DNA-alkylating properties of platinum based chemotherapies, such as carboplatin. Two small format studies have investigated the combination of carboplatin + Docetaxel versus Docetaxel alone with modest but seemingly optimistic results. Twenty percent of Carboplatin + Docetaxel treated patients experienced delayed disease progression with associated PSA decline (Dayyani, Gallick, Logothetis, & Corn, 2011; Nakabayashi et al., 2008; Regan et al., 2010). Disappointingly, after Phase III trials demonstrated no overall survival advantage of satraplatin (oral platinum therapy) in patients who progressed on taxane chemotherapy. Thus, scientific efforts have been redirected towards other evolving therapy options (Sternberg et al., 2009).

Abiraterone Acetate. The COU-AA-301 randomized, double blinded, placebo controlled Phase III clinical trial investigated the effect of Abiraterone acetate in mCRPC patients who had previously received Docetaxel treatment and found an improvement in overall survival of 4.6 months compared to control-treated patients (De Bono et al., 2011; Fizazi et al., 2012). In a retrospective study mCRPC patients who had been treated with Enzalutamide alone or in combination with Docetaxel, Abiraterone acetate yielded only modest anti-tumor effects (Loriot et al., 2013; Noonan et al., 2013)

Enzalutamide. The AFFIRM Phase III, double blinded, placebo controlled clinical trial demonstrated that metastatic castration-resistant prostate cancer patients, previously treated with taxanes, were definitively responsive to enzalutamide (Hoffman-Censits & Kelly, 2013; Scher, Fizazi, Saad, Taplin, Sternberg, Miller, De Wit, Mulders, Chi, et al., 2012). Patients treated with enzalutamide had an overall survival of 18.4

months versus 13.6 months for the placebo control treated patients. Furthermore, enzalutamide-treated patients who had reduction in PSA level by 50% or greater had improved quality of life, longer radio-graphic progression free survival, and increased time to first skeletal related event (Scher, Fizazi, Saad, Taplin, Sternberg, Miller, De Wit, Mulders, Chi, et al., 2012). In a direct comparison trial, the efficacy of enzalutamide was shown to be greater when administered to Docetaxel naïve patients as opposed to those pre-treated with taxane (Nadal et al., 2014). In contrast, the use of enzalutamide in patients with metastatic CRPC pre-treated with Docetaxel and Abiraterone, was not overtly effective. Specifically, in 39 patients with metastatic CRPC, selected for a retrospective analysis, 41% of patients had a PSA decline of 30% or greater, but overall activity of enzalutamide in this population was fairly limited (Bianchini et al., 2014). Despite the small sample size, this study implicates that mechanisms of cross resistance are at play between these three agents when they are sequentially administered. Another recent study confirmed these results by showing that enzalutamide exhibited limited activity in mCRPC patients previously treated with Docetaxel and Abiraterone, but for those patients which exhibited PSA decline of greater than 30% overall survival was significantly improved (Brasso et al., 2014).

Radio-pharmaceuticals such as ^{153}Sm-ethylenediaminetetramethylenephosphonate (^{153}Sm –EDTMP) may offer a unique combination with Docetaxel. This therapy is intravenously administered and delivers β-emitting radiation to newly remodeled bone such as osteoblastic bone metastases (Goeckeler et al., 1987; Madan et al., 2011) . This therapy has been shown to confer palliation of bone pain in mCRPC patients and has been approved by the FDA for such use (O. Sartor et al., 2004; Serafini et al., 1998). Promising Phase I trial results indicated

that the combination therapy of ^{153}SmEDTMP and Docetaxel was well tolerated and induced decline in PSA level (Serafini et al., 1998).

Clusterin targeting (OGX-011). Clusterin is also known as testosterone-repressed prostate message 2 and sulfated glycoprotein-2 (Gleave & Miyake, 2005; Zellweger et al., 2002). Functionally, clusterin is involved in tissue remodeling, reproduction, lipid transportation, apoptosis, and more (Dayyani et al., 2011). Its association with apoptosis has been controversial because it is highly overexpressed in dying tissues, but contradictorily it is also highly overexpressed in prostate, lung, breast tumors, lymphoma, and renal cell carcinoma (Gleave & Miyake, 2005). Clusterin becomes highly expressed in castration and chemo- resistant prostate tumors rendering it a potential candidate for targeting. In fact, induced overexpression of clusterin can confer resistance to hormone deprivation and taxane chemotherapy in LNCaP xenografts tumors (Gleave et al., 2001). In order to target clusterin, employment of an antisense oligonucleotide to the clusterin gene was developed and optimized to become Custirsen/ OGX-011 (Gleave et al., 2001). A randomized phase II trial investigated the effect of Docetaxel + Prednisone with or without OGX-011 in 82 metastatic CRPC patients (Chi et al., 2010). Although, there was no difference in PSA decline between the two arms, this trial provided encouraging results in that OGX-011 was well tolerated in patients and demonstrated improved overall survival compared to those treated with Docetaxel + Prednisone alone (23.8 versus 19.6 months) (Chi et al., 2010). Another randomized phase III trial concluded that tested Docetaxel + prednisone versus Docetaxel + prednisone with OGX-011 in nearly 1,000 patients with overall survival as the primary endpoint (Dayyani et al., 2011).

Other combinations. Extensive efforts have been invested in identifying potential efficacious for combination therapy with taxane chemotherapy, but disappointment has

been rampant (Antonarakis & Eisenberger, 2013). Antiangiogenic agents including Bevacizumab and Aflibercept in combination with Docetaxel did not confer any improvement in overall survival (Fizazi et al., 2013; Tannock et al., 2013) . Other agents targeting the bone microenvironment have been tested in phase II trials; Atrasentan, Zibotentan, and Dasatinib among them did not garner overall survival benefit when combined with Docetaxel compared to Docetaxel alone (Araujo et al., 2013; Kelly et al., 2012; Quinn et al., 2012). Furthermore, immune modulators, GVAX and Lenalidomide actually produced inferior overall survival results compared to Docetaxel alone (Petrylak et al., 2012; Small et al., 2009).

Docetaxel to Cabazitaxel. The TROPIC phase III clinical trial led to FDA approval of Cabazitaxel (Jevtana, Sanofi-Aventis) for use in mCRPC patients who progressed on Docetaxel chemotherapy (De Bono, Oudard, Ozguroglu, Hansen, Machiels, Kocak, Gravis, Bodrogi, Mackenzie, Shen, Roessner, Gupta, Sartor, et al., 2010). The trial demonstrated a significant overall survival benefit for patients treated with Cabazitaxel versus mitoxantrone of 2.4 months. Cabazitaxel-treated patients had a 30% relative reduction in risk of death, doubled rate of progression-free survival, and demonstrated both PSA and tumor response compared to mitoxantrone treated patients (De Bono, Oudard, Ozguroglu, Hansen, Machiels, Kocak, Gravis, Bodrogi, Mackenzie, Shen, Roessner, Gupta, Sartor, et al., 2010). One must critically consider the recently demonstrated therapeutic impact of Cabazitaxel in patients progressing on Docetaxel followed by Abiraterone acetate as well as Docetaxel followed by enzalutamide. This clinical evidence supports an action by Cabazitaxel not directly targeting the AR signaling axis, as established for Docetaxel (Nakouzi et al., 2014; Pezaro et al., 2014; M.-L. Zhu et al., 2010a), thereby challenging its place in limitations of overcoming cross-resistance with anti-androgen treatment regimes in CRPC (Figure 1.6).

Conclusions

The AR acts as a cornerstone of the aberrant signaling mechanisms associated with prostate cancer. Intense pursuit of the anomalous pathways via which androgen signaling is perpetuated in CRPC has identified “diverting” mechanisms that still impact tumor progression and therapeutic response in patients. The androgenic signaling axis can become altered in a number of ways: point mutations, truncations, variant expression of the AR itself, post translational modifications deviating from the normal signaling by RTKs and downstream of growth factor signaling pathways, and the ability of prostate cancer cells to commandeer androgen synthesis in the face of ADT. In close exchanges directed by AR, EMT can be reactivated in prostate cancer epithelial cells by the key signaling controllers of prostate growth and their functional interactions (TGF-β and androgen axis/AR), towards metastatic behavior. Thus, unfolding the key players in EMT-activating signaling pathways engaged in crosstalk with AR signaling is paramount to recognizing potential therapeutic targets for CRPC. Loss of E-cadherin expression and induction of N-cadherin are regulated by key transcription factors, Snail and Slug, which transcriptionally repress E-cadherin via the androgenic signaling axis. In a more prominent role, Zeb1, directly recruited by the AR signaling, engages in a bidirectional negative feedback loop, highlighted in ADT. In the absence/repression of AR (as in early ADT), Zeb1 is overexpressed facilitating the mechanistic events leading to EMT. Also impacted by the androgenic status, β-catenin, accumulates in the cytoplasm and translocates to the nucleus, to induce transcription of LEF1/TCF genes and others which mediate EMT processes. Interestingly, β- catenin can also interact with AR directly and act as a transcriptional coactivator of AR driving not only EMT, but progression to CRPC.

In a less direct crosstalk event, AR drives over-expression of TMPRSS2: ERG genes fusion products resulting in highly overexpressed transcription factor ERG (ETS

family of transcription factors). In the clinical setting, overexpression of ERG and FZD is associated with prostate tumor progression. At the cellular level, elevated ERG induces EMT, an effect that can be abrogated by silencing of FZD, thus implicating the Wnt signaling pathway in driving the effects of ERG gene fusions (Gupta et al., 2010). In view of the documented significant association between elevated TGF-β ligand and increasing prostate tumor grade, a dynamic cross-talk of TGF-β signaling with AR, in controlling EMT during progression to metastasis, becomes central to the cellular landscape of CRPC development. Moreover, Smads3 and 4 interact directly with AR to reciprocally modulate both target gene transcriptional activation and expression. Androgen treatment of human prostate cancer cells significantly upregulates Snail and promotes the TGF-β and AR interaction in controlling EMT (M. Zhu & Kyprianou, 2010). Notch signaling is essential to intercellular communication, and expression of Jagged1 (main effector) emerges as a potential independent prognostic indicator of prostate cancer recurrence and progression, since expression of Jagged1 correlates with high grade and metastatic prostate cancer compared to benign disease or localized tumors. Moreover Jagged1 bypasses AR in prostate cancer metastasis, and Notch1 signaling is functionally involved with osteoblast differentiation in skeletal derived prostate cancer cells. As our understanding of the role of AR signaling in navigating EMT towards prostate cancer metastasis and CRPC expands, so do the opportunities to exploit the interactions of AR with lead partners, in pursuit of novel therapeutic targets and prognostic indicators of disease progression.

Rapidly growing evidence supports additional actions of taxanes beyond microtubule stabilization. Docetaxel exerts many effects on prostate tumor cells including increasing expression of FOXO1, inhibiting translocation of the AR from the cytoplasm to the nucleus, and overcoming overexpression of Bcl2 (Haldar, Basu, & Croce, 1997) .

While Cabazitaxel treatment exerts a similar mechanism of “classic” microtubule targeting action, additional mechanisms also emerge that may drive a new therapeutic response to this 2nd line chemotherapy. It regulates the expression of AR and mitotic kinesins MCAK and HSET towards promoting apoptosis. The major challenge of prostate cancer chemotherapy is overcoming inevitable resistance to taxanes caused by alterations in microtubule dynamics, isotype expression, and microtubule associated protein expression. Intermittent taxane chemotherapy and combination therapy with anti-androgens, Enzalutamide and Abiraterone Acetate, have provided additional survival benefit to patients but we are still far from a cure. Emergence of Cabazitaxel as a taxane effective in the post-Docetaxel landscape represented a huge step forward to treatment of CRPC conferring a significant improvement in survival for patients. Both of these concepts are pursued in the two subsequent chapters of this thesis.

Figure 1.1 The Androgen Receptor (AR)

A, AR Structure: Schematic diagram of protein domains and functions. The N-terminal domain possesses the Tau-1 and Tau-5 transcriptional activation domains which together comprise the AF1 (activation function 1 domain). The DNA binding domain confers AR dimerization and binding to ARE via two zinc finger domains (P-Box and D-Box), which facilitate interaction with the DNA double helix. The Hinge region contains the nuclear localization sequence (NLS), which confers nuclear import and regulates spacing between the NTD and ligand binding domain. The LBD interacts with DHT, associates with chaperones and co-regulatory proteins and interacts with the N terminal domain to achieve AR dimerization. The LBD comprises the second activation function domain (AF2).

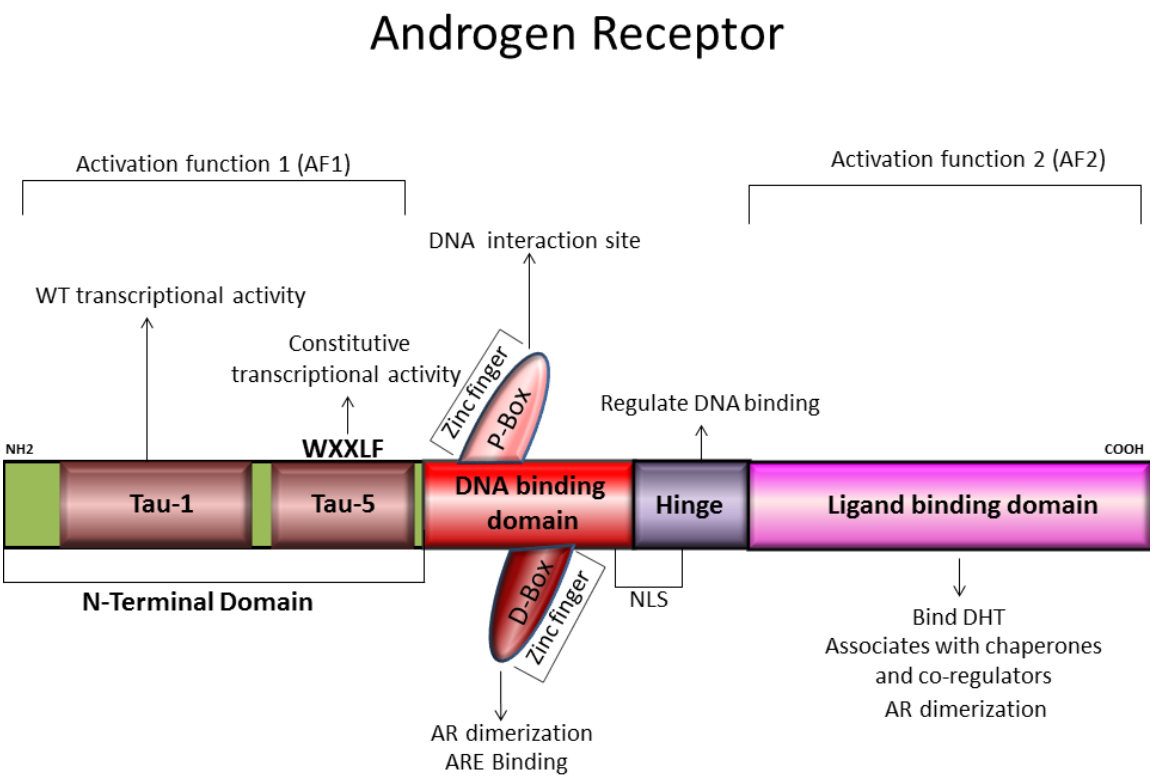


Figure 1.1 continued, The Androgen Receptor (AR)

B, Targeting AR translocation to the nucleus in prostate cancer. A. After binding of cognate ligand, molecular handling by super-chaperone complexes, dimerization, phosphorylation and association with dynein motor proteins onto microtubules (MT), AR translocates to the nucleus to mediate transcription of Androgen Responsive Elements (ARE). B. Taxane chemotherapeutics stabilize the interaction of β-tubulin subunits within the proto-filaments of the microtubule preventing the de-polymerization of the structure resulting in G2M arrest, apoptosis, and increased accumulation of AR in the cytoplasm. C. With regard to Docetaxel (Taxane) resistant – CRPC, differential signaling is potentially inclined to facilitate translocation of AR to the nucleus despite microtubule stabilization.

B.

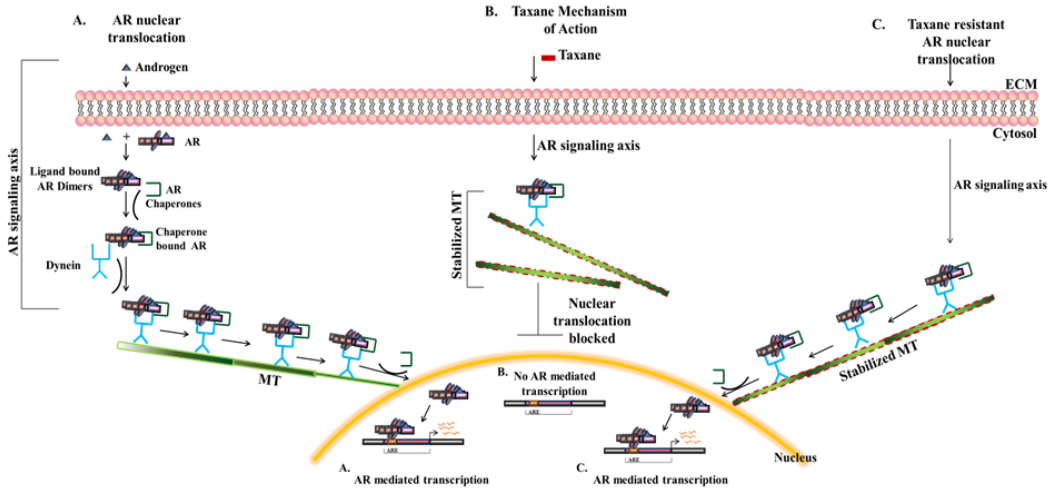


Figure 1.2. Role of AR in prostate cancer and EMT Panel A. Cadherin switching, Zeb1 feedback loop, and expression of Snail2 are modulated by the crosstalk with the Androgen Receptor (AR).

A.

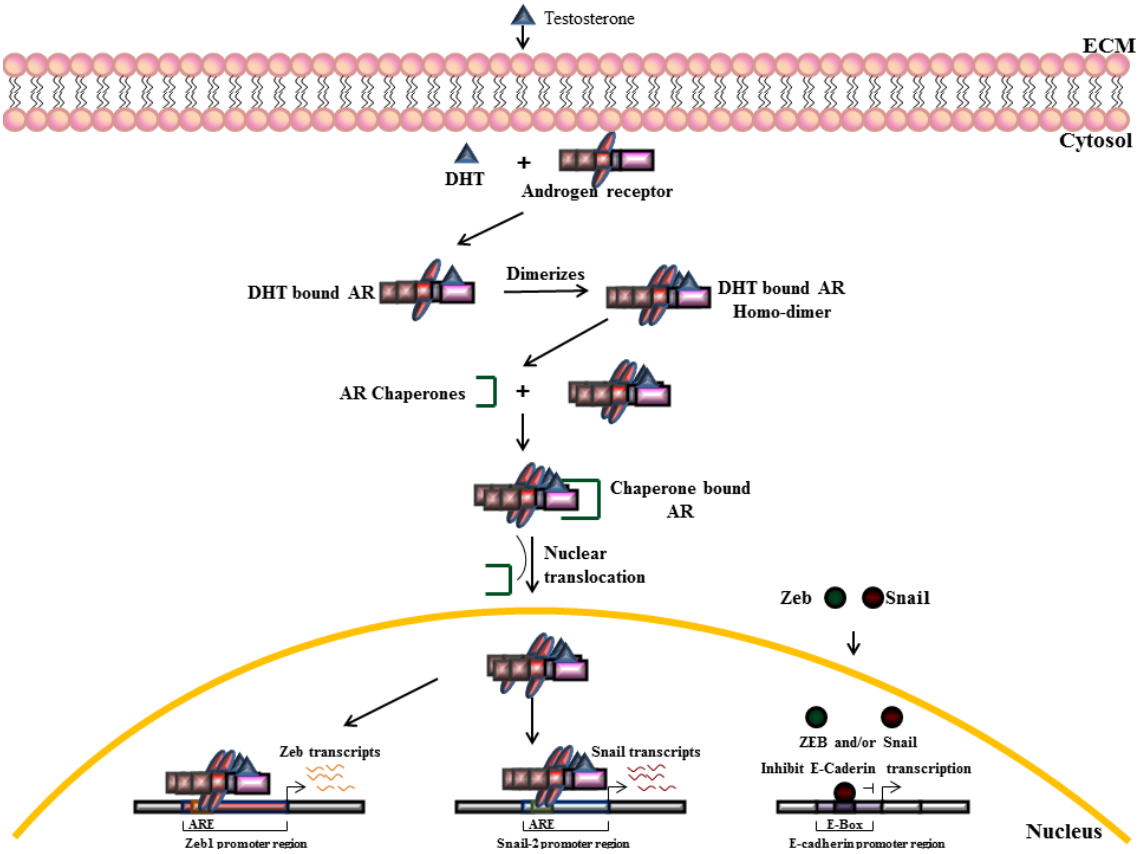
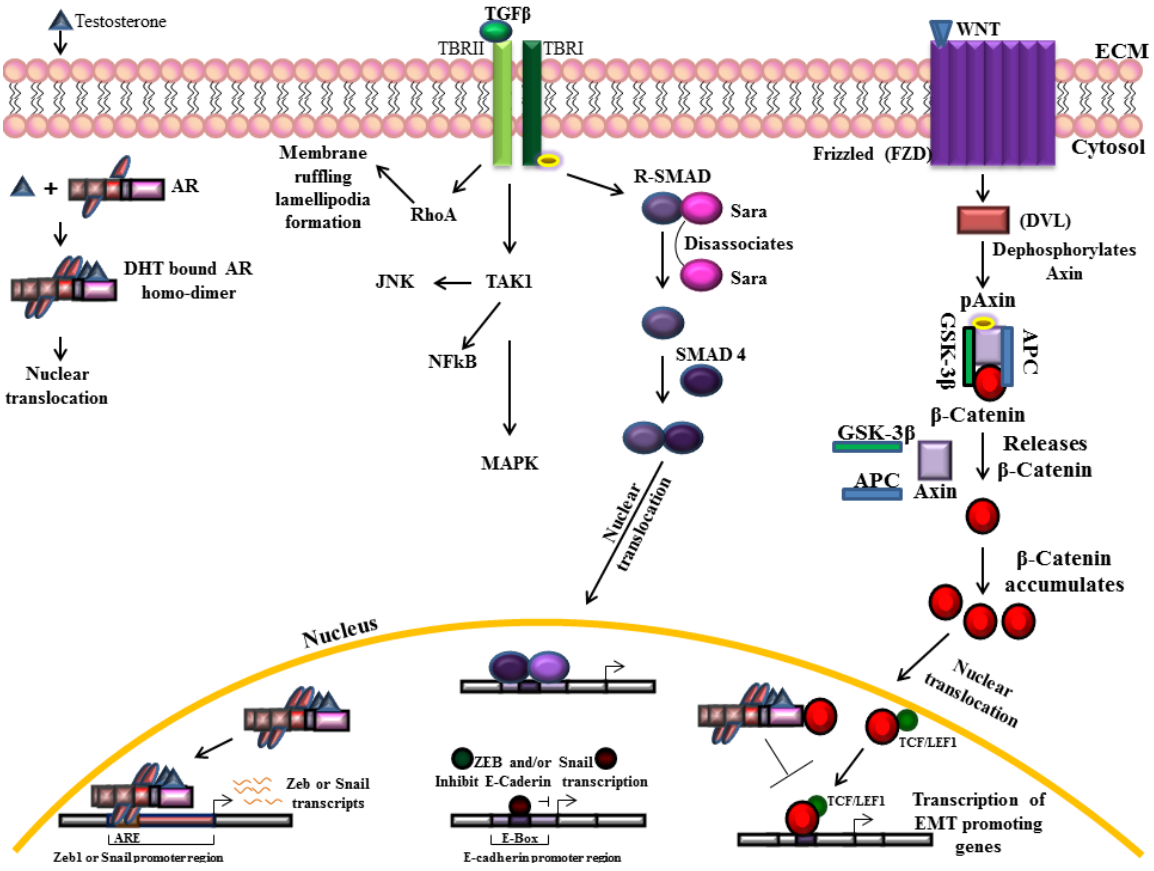


Figure 1.2 continued, Role of AR in prostate cancer and EMT Panel B. TGFβ signaling induces EMT-associated changes in prostate cancer cells via Smad-dependent and -independent signaling and their signaling crosstalk with the AR. C. Wnt/ β-catenin signaling engages in direct crosstalk with AR. AR and TCF-LEF1 transcription factors compete for β-catenin acting as a coactivator of transcription promoting EMT.

B.



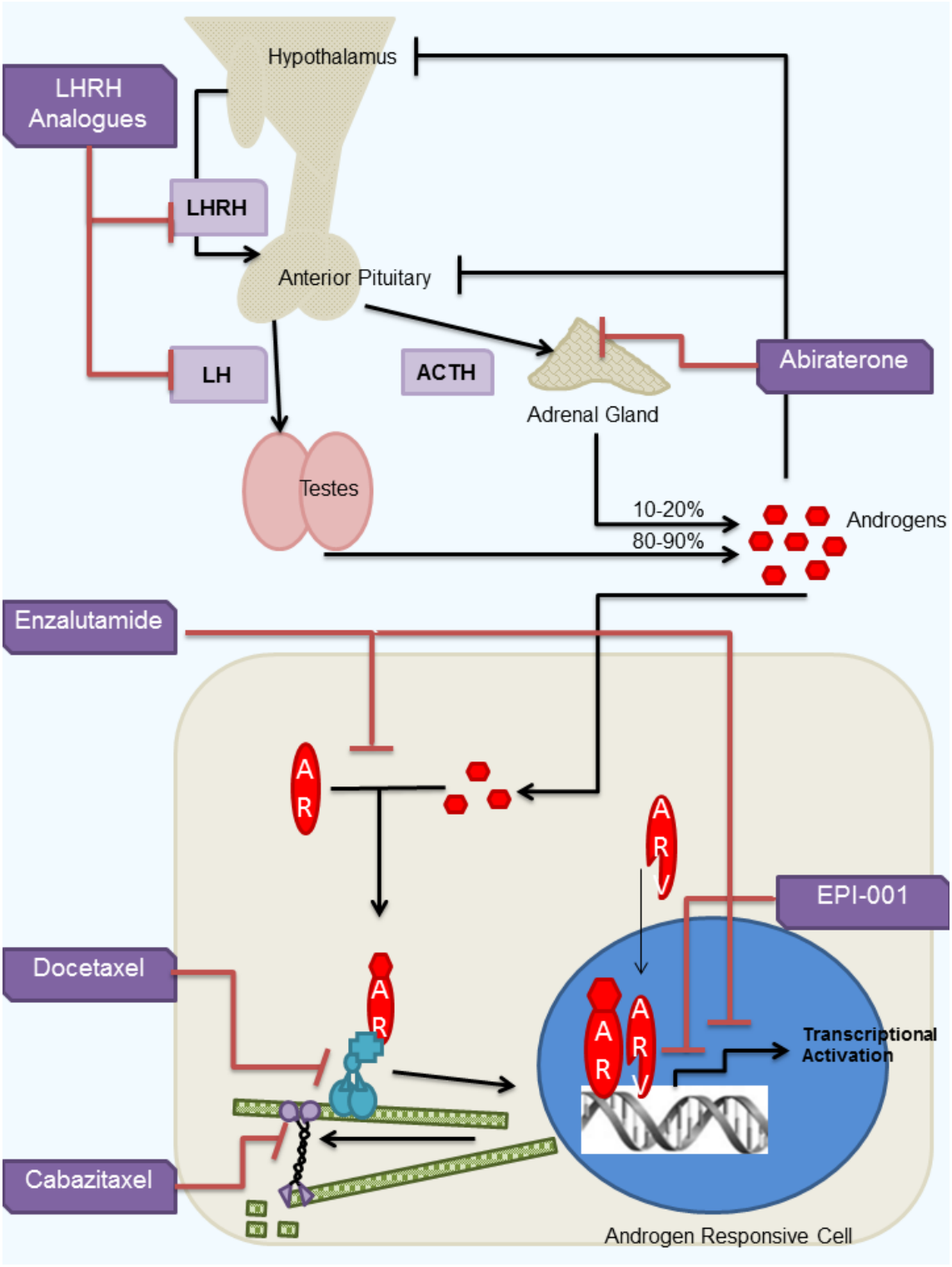

Hypothalamus
LHRH Analogues
LHRH
Anterior Pituitary
LH
ACTH
Abiraterone
Adrenal Gland
Testes
10-20%
80-90%
Androgens
Enzalutamide
AR
ARV
EPI-001
Docetaxel
Transcriptional Activation
Cabazitaxel
Androgen Responsive Cell

Figure 1.3 Therapeutic Targeting of Hormonally Dependent Prostate Cancer. Therapies employed to treat prostate cancer include inhibiting signaling through the hypothalamic pituitary axis via LHRH Analogues, inhibition of adrenal androgen synthesis with Abiraterone, direct inhibition of AR activity by treatment with AR antagonist ,Enzalutamide, targeting of the NTD of AR with EPI-001 to prevent transcriptional activation, microtubule stabilization by Docetaxel leading to inhibition of dynein mediate AR nuclear translocation and Cabazitaxel mediated microtubule (MT) stabilization leading to AR stranded in the nucleus and decreased expression of MT de-polymerizing kinesins.

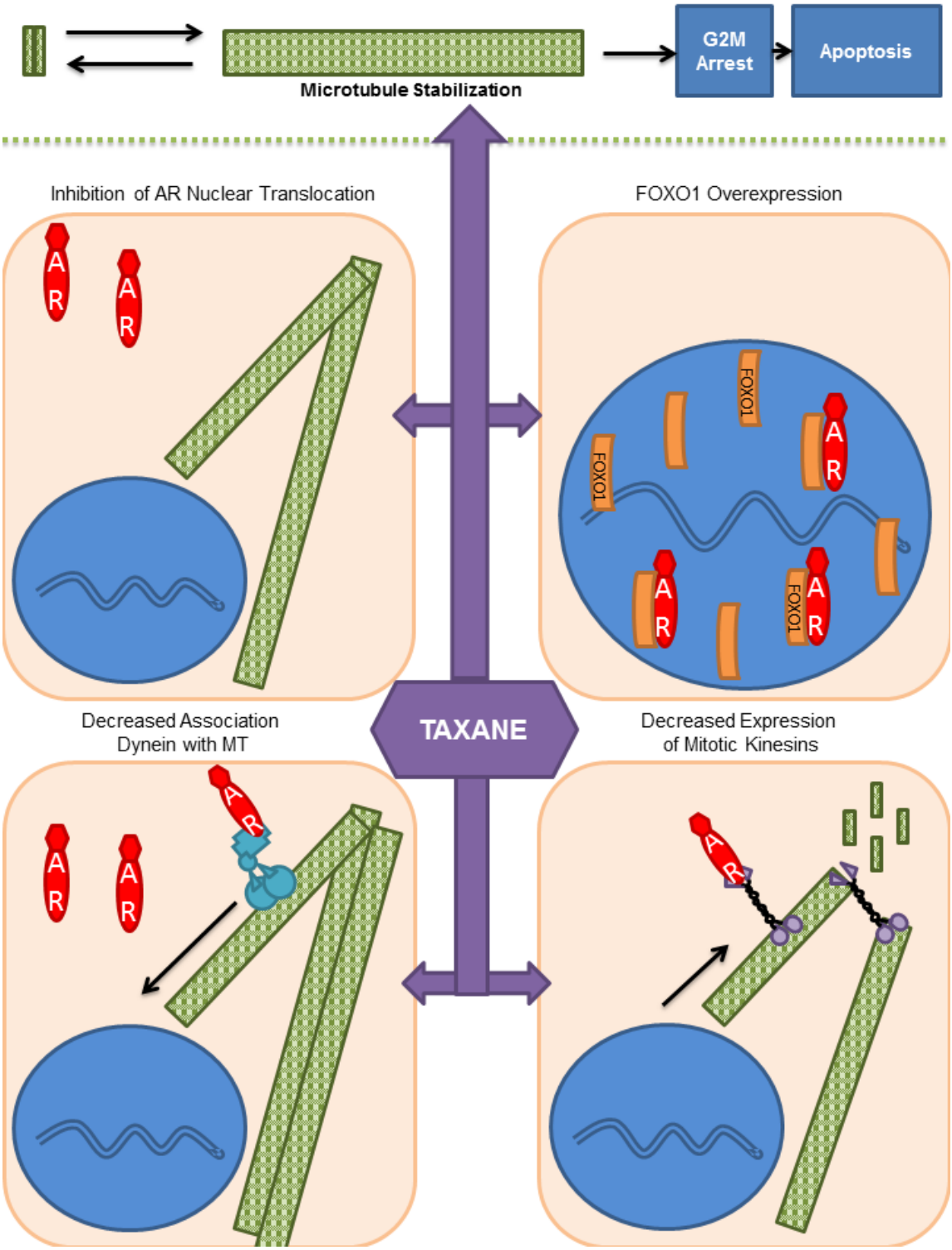

Microtubule Stabilization
G2M Arrest
Apoptosis
Inhibition of AR Nuclear Translocation
FOXO1 Overexpression
TAXANE
Decreased Association Dynein with MT
Decreased Expression of Mitotic Kinesins
AR
FOXO1

Figure 1.4 Taxane Mechanism of Action. Taxanes (Docetaxel and Paclitaxel) directly stabilize the interaction between tubulin subunits to prevent de-polymerization of microtubules (MT), leading to G2M arrest and apoptosis. In addition, taxanes can inhibit AR nuclear translocation along the MT, induce overexpression of FOXO1, and thus causing sequestration of AR away from ARE. Taxanes also inhibit the association of dynein with microtubules (MT) leading to diffuse AR localization. Moreover, Cabazitaxel therapy decreases expression of pro-mitotic kinesins in prostate cancer cells.

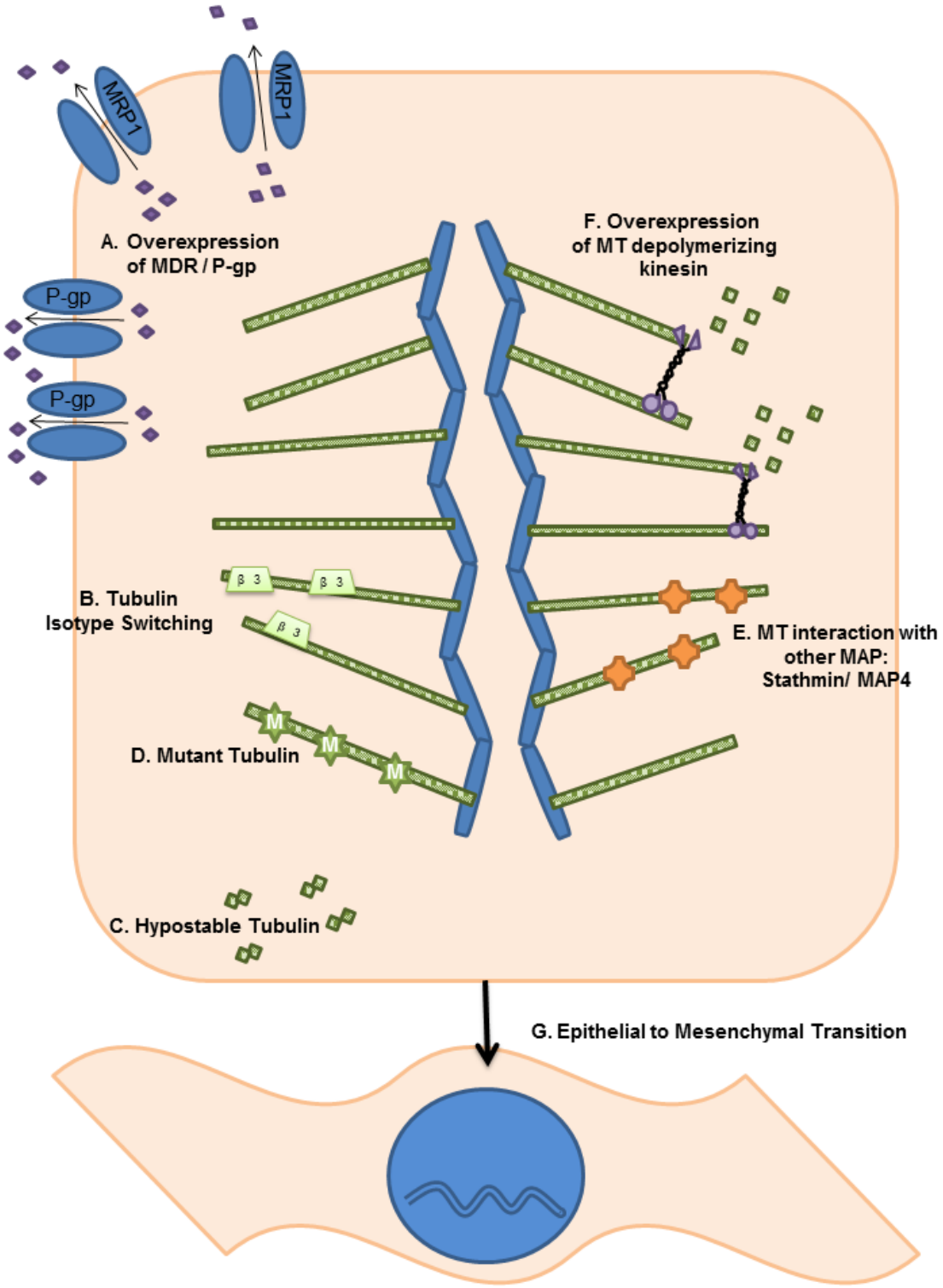
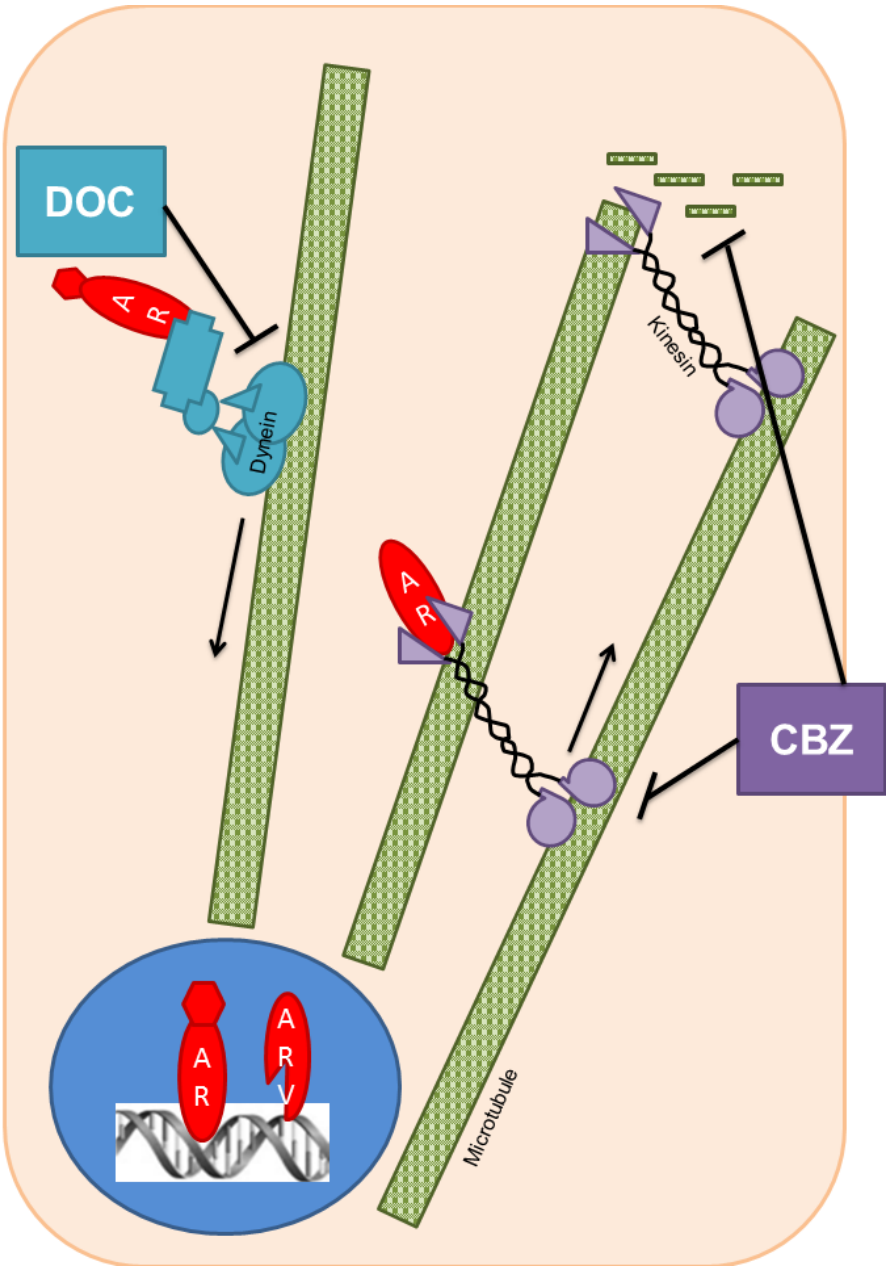

MRP1
MRP1
A. Overexpression of MDR / P-gp
P-gp
P-gp
F. Overexpression of MT depolymerizing kinesin
B. Tubulin Isotype Switching
β 3
β 3
β 3
E. MT interaction with other MAP: Stathmin/ MAP4
M
M
M
D. Mutant Tubulin
C. Hypostable Tubulin
G. Epithelial to Mesenchymal Transition

Figure 1.5 Mechanisms of Taxane Resistance in CRPC

Overexpression of MDR1/P-gp ATP dependent drug efflux pumps can promote resistance by increasing the efficiency with which cells may pump out taxanes Panel (A), Alterations in the tubulin subunit and its structural/spatial dynamics leads to taxane resistance via isotype switching from βI to βIII tubulin. Panel (B); expression of tubulin with a hypostable binding site leads to a shift in microtubule (MT) binding dynamics towards dimerized tubulin and away from polymerized tubulin; Panel (C), mutational alterations in the tubulin subunit lead to decreased binding of Docetaxel to microtubule structure, consequently conferring resistance to taxane. MT interaction with other microtubule associated proteins (MAP) such as MAP4 and Stathmin affect stability of MT structure (E). Overexpression of MT depolymerizing kinesins (HSET, MCAK, Eg5) can lead to taxane resistance by circumventing effects of stabilized MT (F). Induction of EMT also causes resistance to taxane therapy (G).

Figure 1.6 Differential Effect of Docetaxel (DOC) and Cabazitaxel (CBZ) on ATP-dependent Motor Proteins Driving Therapeutic Response. Docetaxel decreases the association between AR and dynein thereby inhibiting the translocation of AR from cytoplasm to nucleus. Cabazitaxel therapy decreases expression of microtubule de-polymerizing kinesins (MCAK and HSET), causing multi-nucleation and centrosomal amplification stranding AR in the nucleus.



Chapter 2. N-Terminal Targeting of AR Variant Enhances Response of Castration Resistant Prostate Cancer to Taxane Chemotherapy

Background and Significance

Development of metastatic castration-resistant prostate cancer (mCRPC) is a consequence of lack of an apoptotic response to androgen deprivation (B. J. Feldman & D. Feldman, 2001). The treatment landscape for mCRPC has been transformed by the recent FDA approval of the androgen/AR signaling axis inhibitors, built on clinical evidence that AR signaling drives both the therapeutic response and resistance in mCRPC (C. D. Chen et al., 2004; Visakorpi et al., 1995). Overexpression of AR, detected in CRPC, (B. J. Feldman & D. Feldman, 2001; Visakorpi et al., 1995) is able to mediate resistance to anti-androgens (C. D. Chen et al., 2004), while point mutations increasing the ligand-binding affinity of AR cause signaling hypersensitivity (Gregory et al., 2001). Promiscuous mutations cause binding flexibility in the ligand-binding domain (LBD) allowing the AR to become activated by adrenal androgens, androgenic metabolites, and anti-androgen therapeutics including enzalutamide and ARN-509 (Dehm et al., 2008; Scher, Fizazi, Saad, Taplin, Sternberg, Miller, De Wit, Mulders, Hirmand, et al., 2012; Tanner et al., 2010; Yilmaz & Christophori, 2009). Moreover, over twenty splice variants of AR, some lacking LBD, and therefore constitutively active have been identified and associated with progression of CRPC and metastasis (Dehm et al., 2008; Z. Guo et al., 2009; Hu et al., 2009; Hu, Isaacs, et al., 2010; Jenster et al., 1995; S. Sun et al., 2010). AR signaling mediated by truncated AR splice variants (AR-Vs) lacking LBD, may potentially drive the emerging resistance to anti-androgen therapies and CYP17 inhibitors (B. Cao et al., 2014; Mostaghel et al., 2011; X. Zhang et al., 2011). In support of this, selective loss of AR V7 variant in prostate cancer cells restored sensitivity to enzalutamide (Y. Li et al., 2013). Furthermore, expression of an androgen

receptor variant can predict therapeutic response to anti-androgens such as enzalutamide (Antonarakis et al., 2014).

Taxane-based chemotherapy is a clinically effective treatment for CRPC, by disruption of microtubule dynamics via stabilization of β-tubulin subunits within the microtubule structure, resulting in deregulation of the mitotic spindle assembly. As discussed in the introduction, taxanes bind the β-subunit of tubulin, stimulating polymerization into stabilized microtubules that inhibit cell cycle progression leading to G2M arrest and apoptosis (Harrington & Jones, 2011; Vrignaud et al., 2013). Evidence from this laboratory and others established that taxane stabilization of microtubules inhibits AR translocation into the nucleus thus, preventing the transcriptional activity of AR (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a). Additionally, taxanes lead to an increase in Forkhead box 01 (FOXO1), a transcriptional repressor of AR, consequently resulting in inhibition of ligand-dependent and ligand-independent transcription (Gan et al., 2009a). The therapeutic impact of Docetaxel in prohibiting prostate cancer progression and improving survival in patients with advanced disease, has been attributed to utilization of mechanisms previously targeted by androgen deprivation therapy (ADT) (Fitzpatrick & de Wit, 2014; Mistry & Oh, 2013). Despite a proven survival advantage, resistance to Docetaxel develops, leading to disease progression in approximately 7.5 months (Loriot & Fizazi, 2013). Mechanisms implicated in the development of Docetaxel resistance include overexpression of P-glycoprotein drug efflux pump, mutational alterations in tubulin expression and induction of EMT (Fitzpatrick & de Wit, 2014; Loriot & Fizazi, 2013; Puhr et al., 2012).

The N-terminal domain (NTD) of AR is effectively targeted by the novel small molecule, EPI-001/002 (EPI), which interacts with the disordered domain of the AF-1

region and blocks AR transcriptional activity (Andersen et al., 2010; Myung et al., 2013). The functional contribution of the microtubule network and the cytoskeleton to androgen-mediated signaling via navigating AR cellular localization, as well as the consequences of their inhibition by taxanes on AR activity in human prostate cancer have been established (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a). Considering the compelling evidence that AR variant expression and EMT have both been implicated as mechanisms of resistance to anti-androgens and taxane-based chemotherapy and poor survival (Hornberg et al., 2011; Y. Li et al., 2013; Mostaghel et al., 2011; Puhr et al., 2012; X. Zhang et al., 2011) and since the association of AR with tubulin occurred via the AR NTD (M.-L. Zhu et al., 2010a), this study investigated the effect of the novel AR NTD antagonist EPI on the sensitivity to taxane treatment *in vitro* and *in vivo* of CRPC harboring the full length AR and the AR splice variants. My work identified that such a combination strategy effectively suppressed CRPC via navigating cycles of EMT and changes in the cytoskeleton integrity.

Approach

Cell Lines. The human prostate cancer cell lines, the castration-resistant cell line 22Rv1 and androgen-sensitive LNCaP cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (FBS), 100units/ml penicillin and 100µg/ml streptomycin in a 5% CO2 incubator at 37C. The androgen-independent LNCaP95 cell line (derivative cells from LNCaP) was a generous gift from Dr. Stephen Plymate (University of Washington, Seattle, WA). For experiments examining responses

to androgen, cells were seeded in 10% charcoal-stripped serum and were stimulated for 24hrs by growth medium containing 1nM dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO) or R1881.

Antibodies. The following antibodies were used for the various experiments. Antibodies against E-cadherin, β-tubulin, Androgen Receptor (N-20), Dynein IC1/2 proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against N-cadherin and CD31 were obtained from AbCam Cell Signaling (Cambridge, UK); antibodies against PARP-1, Vimentin, GAPDH, Snail, cofilin and β-catenin proteins were obtained from Cell Signaling Technology (Danvers, MA).

Cell Viability Assay. The effect of the various treatments on prostate cancer cell viability was evaluated using the Thiazolyl Blue Tetrazolium bromide (MTT) assay). Cells were seeded into 24-well plates, grown to 60-75% confluence, treated with vehicle control (DMSO, Sigma-Aldrich, St. Louis, MO), Docetaxel (DOC, 1μM), EPI-002 (25μm), or the combination (1μM DOC + 25μM EPI-002) in RPMI 1640 with 10% CSS (Charcoal Stripped Serum) for 24hrs. Following treatment, media was aspirated and cells rinsed with PBS then treated with 250μl/well MTT (1mg/ml) for 30mins at 37°C. After incubation, MTT was aspirated and formazan crystal was solubilized with DMSO. Absorbance was measured at 570nm using μQuant Spectrophotometer (Biotech Instruments Inc., Winooski, VT).

Cell Proliferation. Cells were seeded into 96-well plates and pretreated for 1hr with vehicle or EPI-002 (25μM). After 16hrs, cells were treated with Docetaxel (0.5μM) (alone or combination with EPI-002) before addition of R1881 (synthetic androgen, 0.1nM) or vehicle under serum-free and phenol red-free conditions, and subsequently incubated for 23hrs. After pulse-labeling with 10 μM BrdU (for 2hrs), BrdU-labeled cells

were identified with the anti-BrdU-POD (Roche). BrdU incorporation was measured at 570nm using a VersaMax ELISA Microplate Reader (Molecular Devices). The results from three independent experiments performed triplicate were analyzed.

Western Blot Analysis. Total cellular protein was extracted from cell pellets by homogenization with RIPA buffer (Cell Signaling Technology, Danvers, MA). Protein samples were loaded into 4% to 12% SDS-polyacrylamide gels (Bio-rad, Hercules, CA) and subjected to electrophoretic analysis and blotting. The following antibodies were used against these specific proteins: E-cadherin, N-cadherin, β-tubulin, the AR (N-20), Snail, Vimentin, PARP-1, β-catenin and GAPDH proteins. Membranes were incubated with the specific primary antibody (overnight at 4°C) and were subsequently exposed to the relevant secondary antibody (90mins, room temperature). For signal detection, membranes were incubated with the Amersham ECL Plus Western Blotting Detection System (Amersham, GE Healthcare, Buckinghamshire, UK) for 5mins and auto-radiographed using X-ray film (Denville Scientific, South Plainfield, NJ). All protein expression bands were normalized to GAPDH expression (used as loading control).

Quantitative RT-PCR Analysis. *In vitro* samples: RNA was extracted with the Trizol© reagent (Life Technologies, Grand Island, NY) and RNA samples (1μg) were subjected to reverse transcription using the Reverse Transcription System (Promega, Madison, WI). TaqMan real time reverse transcriptase-PCR (Life Technologies, Grand Island, NY) analysis of the cDNA samples was conducted in an ABI7700 Sequence Detection System (Applied Biosystems, Inc, Branchburg, NJ), using the following specific primers: for Prostate Specific Antigen (KLK3; Hs02576345_m1), E-cadherin (CDH1; Hs01023894_m1), N-cadherin (CDH2; Hs00983056_m1), Vimentin (VIM; Hs00185584_m1), Snail (SNAI1; Hs00195591_m1), Twist (TWIST1; Hs01675818_s1), UGT2B17 (Hs00854486_sH), and 18S rRNA (4319413E) (Applied Biosystems, Life

Technologies, Grand Island, NY). For the qRT-PCR experiments, each sample was analyzed in duplicate and data represent average values from three independent experiments. Numerical data for transcript levels were normalized to 18s rRNA in controls and expressed relative to untreated controls.

Luciferase Reporter Gene Assays. LNCaP95 cells (1.5x105) and 22Rv1 cells (9.5x105 in 10% FBS phenol red free RPMI) were transfected with the AR responsive PSA(6.1kb)-luciferase, probasin (PB)-luciferase and ARR3-luciferase reporters in serum-free media. At 5hrs post- transfection, cells were pretreated with vehicle or EPI-002 (EPI) (25μM) for 16hrs, and were subsequently exposed to Docetaxel (DOC) (0.5μM), or EPI and Docetaxel (EPI + DOC). Cells were incubated with R1881 (1nM) for 23hrs. After 24hrs of treatment, cells were lysed and subjected to luciferase reporter activity analyses that were normalized to protein concentrations using the Glomax luminometer (Promega Corporation; Madison, WI). All transfection assays were performed in at least three independent experiments in triplicate wells.

Immunofluorescent Confocal Microscopy. Cells were plated ($1x10^5$) in chamber slides coated with fibronectin (Invitrogen). After 24-48hrs, cells were exposed to medium (RPMI 1640 with 10% CSS) in the presence of DHT (1nM), Docetaxel (DOC: 1μM), EPI-002 (EPI: 25μM) or in combination of the two agents. Following treatment, cells were fixed in 4% (v/v) paraformaldehyde and permeabilized with 0.1% Triton X-100 in sterile phosphate buffer saline (PBS). Fixed cells were incubated overnight with primary antibody specific for AR (N-20), Dynein IC1/2 and Tubulin, (at 4°C) with gentle rocking and the appropriate Alexa-Fluor (Life Technologies, Grand Island, NY) fluorescent secondary (1.5 hrs, room temperature). Slides were mounted using Vectashield mounting medium with DAPI and were visualized using a FV1000 Confocal Microscope (Markey Cancer Center Core, University of Kentucky).

In Vivo Tumor Targeting Studies. All animal experiments were performed in accordance with the guidelines approved by the Animal Care and Use Committee of the University of British Columbia. NOD-SCID mice (6-8wks old) were castrated before any drug treatments. At 7 days post-castration, mice were subcutaneously injected with 22Rv1 cells ($3x10^6$cells plus Matrigel™ media). Mice were subsequently divided into two groups of 10 mice each: the control group receiving 1% medium /0.1% Tween-20 daily via oral gavage; the other 10 mice in the treatment group received EPI-001 (100 mg/kg twice daily) via oral gavage. EPI-001 had no effect as a monotherapy so when tumors reached approximately $140mm^3$, 5 mice in each group was intraperitoneal injected (I.P.) with Docetaxel (15mg/kg) on day 1 and day 5; and the combination treatment of EPI-001 (100 mg/kg twice daily) and Docetaxel (15mg/kg) for 11 days; Tumors were measured twice a week and the volume calculated by using the formula, length x height x width x 0.5236. Prostate tumor xenografts were harvested two days after the last treatment and tissue specimens were subjected to histopathological analysis. Immunostaining was conducted for the expression and cellular localization of AR, cytoskeleton organization, EMT, vascularity (CD31), and apoptosis (TUNEL, EMD Millipore, Billerica, MA). TUNEL analysis for detection of apoptotic cells was performed as previously (Pu et al., 2009).

Immunohistochemical Analysis. Tissue specimens from human prostate tumor 22Rv1 xenografts were formalin fixed and paraffin-embedded; serial sections (5μ) were subjected to immunohistochemical analysis using antibodies against E-cadherin, N-cadherin, β-tubulin, AR (N-20), cofilin, and CD31. After blocking nonspecific binding (1.5%NGS/TBS-Triton), sections were incubated with primary antibody (overnight, 4°C). and subsequently incubated with biotinylated goat anti-rabbit IgG (2hrs, room temperature) and horseradish peroxidase-streptavidin (EMD Millipore, Billerica, MA). Color detection was achieved with SigmaFast 3,3'-Diaminobenzidine tablets (Sigma-

Aldrich, St. Louis, MO) and counterstained with haematoxylin. Images were captured via light microscopy (40x and 100x) using an Olympus BX51 microscope (Olympus America, Center Valley, PA). Content and intensity of immunoreactivity were recorded by two independent observers (S.K.M. and N.K.).

Statistical Analysis. Student's t test or one-way ANOVA were performed using Graph Pad Prism 6 software to determine the statistical significance of difference between means. All numerical data are presented as Mean ± Standard error of the mean (SEM). Statistical significance was set at P value < 0.05. ImageJ software was used for calculation of densitometry.

Results

Combination of Docetaxel and EPI Maximizes Blockade of AR Activity and Impairs Human Castration Resistant Prostate Cancer Growth *In Vitro* and *In Vivo*

Considering that constitutively active AR splice variants V7 and V567es (Hornberg et al., 2011) regulate the expression of both canonical and a unique subset of gene targets that are enriched for M-phase cell cycle genes (B. Cao et al., 2014; S. Sun et al., 2010), I used two different human prostate cancer cell lines as models. They express full length functional AR and AR variants to examine the anti-tumor effect of combined targeting of microtubules and AR NTD. The 22Rv1 human prostate cancer cells express both functional full-length AR with duplication of exon 3 and substantial levels of constitutively active AR-V7, although they do not exhibit a proliferative response to androgens (Dehm et al., 2008; Z. Guo et al., 2009; Marcia et al., 2010). Docetaxel treatment (1μM) of 22Rv1 cells for 24 hrs resulted in significant loss of cell viability (Figure 2.1.A). Combination of EPI with Docetaxel increased loss of cell viability beyond

DOC alone (P<0.05), while EPI as a single agent exerted no significant effect on 22Rv1 cell viability at 24 hours.

To examine the anti-tumor effect of EPI alone or in combination with the microtubule-targeting chemotherapy *in vivo*, I used 22Rv1 xenografts in mice (castrated for 2 -weeks prior to inoculation). As shown in Figure 2.1.B, daily treatment with the AR N-terminal inhibitor EPI (200mg/kg), did not result in a significant growth inhibition of the 22Rv1 xenografts compared to vehicle control. The combination treatment of Docetaxel and EPI led to a significant suppression of CRPC tumor growth compared to either single treatment modality or untreated controls (Figure 2.1.B) (**, P<0.05). This dramatic reduction in prostate cancer xenograft growth was associated with a significant increase in the number of apoptotic cells in the combination treated tumor xenografts compared to untreated control or single treatment arms, as evaluated with the TUNEL assay (Figure 2.2.A & Figure 2.2.C). Assessment of the tumor vascularity based on the CD31 immuno-reactivity in xenograft tumors demonstrated a significant decrease in tumor vascularity in response to EPI and Docetaxel combination compared to untreated controls (Figure 2.2.B; Figure 2.2.D).

In response to Docetaxel, there was an apparent decrease in the AR immuno-reactivity in the 22Rv1 CRPC xenografts (Figure 2.3.A). Quantitative evaluation of AR positivity and cellular localization however, did not reveal any significant changes after any of the treatments relative to untreated control (Figure 2.3.B). Treatment of mice with Docetaxel led to reduction in tubulin expression in the prostate tumor xenografts that was further enhanced by the combination treatment of Docetaxel and EPI. Quantitative analysis of cofilin immuno-reactivity revealed a significant decrease of this critical cytoskeleton protein in response to Docetaxel and the combination treatment, compared to untreated control (Figure 2.3.C). To further interrogate these protein changes elicited

by the combination treatment of Docetaxel and the NTD AR targeting, we use confocal microscopy in 22Rv1 cells *in vitro.* As shown on Figure 2.3.D, treatment with Docetaxel alone (for 4hrs), led to a marked reduction in tubulin expression and mitotic arrest (merged image) in CRPC. Prostate cancer cells exhibiting microtubule stabilization appear to have some cytoplasmic AR localization in 22Rv1 cells (Figure 2.3.D and E). Consistent with the *in vivo* xenograft data (Figure 2.3.A), there were no significant changes in the expression of AR after Docetaxel treatment (as single agent or in combination with EPI) compared to untreated control cells (Figure 2.3.D and E).

Docetaxel caused a significant loss in body weight compared to animals treated with vehicle control (86% +/- 3.0% vs 102% +/- 1.3%, p=0.0011), while EPI had no significant effect (98% +/- 1.3%, p=0.6088). The body weight of mice treated with EPI in combination with Docetaxel was also significantly lower than that obtained for the vehicle control (86% +/- 5.4%, p=0.0156), but not significantly different than the weight measurements from mice treated with Docetaxel monotherapy (p=0.9529). Thus the combination treatment of EPI and Docetaxel does not appear to induce toxicity beyond that obtained from Docetaxel alone.

Impact of AR Variants on Taxane-mediated AR Trafficking

The existence of AR variants capable of androgen-independent signaling may be critical in dictating the therapeutic response to taxanes. The AR variant core consisting of the AR NTD and DNA-binding domain is sufficient for nuclear localization and androgen-independent transcriptional activation of AR target genes (S.C. Chan, Y. Li, & S.M. Dehm, 2012). I examined the effect of EPI and Docetaxel on the expression and cellular localization of the AR, as well as tubulin, in the 22Rv1 cells in the presence of DHT. An apparent stabilization of microtubule structures and a decrease in tubulin levels

were detected in response to the combination treatment of Docetaxel and EPI (Figure 2.3.D and E). Figure 2.3 reveals fluorescent images of the 22Rv1 prostate cancer cells in response to Docetaxel or EPI (as single agents), or the combination of the two agents. A modest effect of Docetaxel on androgen-induced nuclear AR translocation was observed after 4hrs of treatment. This was confirmed by subcellular fractionation analysis revealing that Docetaxel treatment reduced the nuclear presence of full length AR, without causing marked changes in AR variants (nuclear vs cytosolic levels) (Figure 2.4.A). This is in contrast to the ability of Docetaxel and also EPI (given as single agents or in combination) to promote the cytoplasmic sequestration of AR from the nuclei in the androgen-sensitive LNCaP cells, harboring a full length AR (Figure 2.5.A). This impact on AR nuclear translocation by taxane and EPI-Docetaxel combination in LNCaP cells was translated into a significant inhibitory effect against cell viability (Figure 2.5.B). Western blot analysis revealed that within 6hrs of treatment with EPI there was an increase in protein levels of both full-length and variant AR compared to untreated 22Rv1 cells that was suppressed by the combination treatment of EPI with Docetaxel (Figure 2.6.B).

Dynein is a microtubule-traversing motor protein capable of efficiently facilitating nuclear transport of cytoplasmic proteins, that was previously shown to navigate AR trafficking along the microtubules and its nuclear translocation, towards induced transcriptional activity (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011). I thus examined the expression and cellular localization of dynein in 22Rv1 cells in response to the various treatments and its co-localization with AR. Androgens (DHT) increase expression of dynein and this correlates with increased AR nuclear translocation (Figure 2.6.A; Merge + DAPI). In response to Docetaxel, there was a marked alteration in dynein localization as detected by confocal microscopy (appearing

as punctate sequestration (Figure. 2.6.A). In marked contrast, in the androgen-sensitive LNCaP cells (full length AR), treatment with Docetaxel alone or in combination with EPI, in the presence of DHT, resulted in diffuse cellular localization of dynein (Figure 2.5.A).

Differential Effect of Taxane and EPI Treatment on AR vs AR-V7 Target Genes

To determine the impact of treatment on the functional activity of AR, the temporal induction of AR regulated genes was evaluated in 22Rv1 cells in response to Docetaxel, EPI or the combination treatment. Prostate-specific antigen (PSA/KLK3) is a gene target for the full length AR and UGT2B17 is a gene target for AR V7 variant. There was a significant increase in KLK3 mRNA expression in response to treatment (within an hour) with Docetaxel monotherapy and combination therapy (Figure 2.6.C) ($P<0.05$). There was no significant effect on KLK3 expression, in response to either EPI or Docetaxel given as single agents, or in combination (after 24 and 48hrs), relative to untreated levels. A transient increase in UGT2B17 mRNA levels was detected within 1-6hrs of treatment with EPI (Figure 2.6.D). After longer treatment periods (12-48hrs), upregulation of UGT2B17 in response to either EPI or combination with Docetaxel, was not sustained.

Next I examined the combination treatment of Docetaxel and EPI on AR transcriptional activity to find it exerts an enhanced inhibitory effect therein, using three AR-driven reporter gene constructs in the 22Rv1 cells. The probasin (PB, -286/+28) reporter gene construct is the natural promoter that contains two functional androgen response elements (AREs), that comprise the androgen response region (ARR). The PSA (6.1 kb) reporter gene construct is also a natural reporter with several AREs in both the enhancer and promoter regions. The ARR3 is an artificial reporter with 3 repeats of the PB ARR in front of a minimal thymidine kinase promoter. The data shown on Figure

2.6, demonstrate that treatment with EPI led to significant inhibition of the androgen-induced PB-luciferase and PSA-luciferase reporters (panels E and F respectively), while unexpectedly increased the activity of the synthetic ARR3 reporter (Figure. 2.6.G) ($P<0.05$); this finding resonates with the increase in AR protein levels by EPI (Figure 2.6.B). The combination of Docetaxel and EPI consistently achieved a significant inhibitory effect for all three AR-driven reporters in 22Rv1 cells (Figure 2.6, panels E-G).

To validate the enhanced anti-tumor potency of the combination approach of taxanes and EPI against another androgen-insensitive prostate cancer cell line, the proliferative response of LNCaP95 human prostate cancer cells that express functional full-length AR and the AR-V7 variant was investigated (Hu et al., 2012). We found that EPI treatment alone or in combination with Docetaxel significantly inhibited cell proliferation in LNCaP95 cells (Figure. 2.7.A). Furthermore analysis of the AR transcriptional activity, demonstrated that treatment of LNCaP95 cells with either EPI or Docetaxel given as single agents significantly inhibited the androgen-induced activity for all three reporters (Figure 2.7, panels B, C and D), prior to manifestation of cell death (Figure 2.8.E). Consistent with the findings in 22Rv1 cells, the combination of Docetaxel and EPI resulted in maximal blockade of AR transcriptional activity in LNCaP95 cells.

Role of EMT in Therapeutic Response of CRPC to Combined Targeting of AR NTD and Microtubules

The recently reported association of AR variants with clinical prostate cancer progression and therapeutic resistance to taxanes and anti-androgens (B. Cao et al., 2014; Y. Li et al., 2013; Mostaghel et al., 2011; X. Zhang et al., 2011) may be driven by induced EMT. To assess the impact of AR NTD targeting on the EMT phenotype of prostate tumor epithelial cells after Docetaxel treatment, we subsequently conducted an

expression profile of key EMT effectors in 22Rv1 cells following single agent or combination treatment. Treatment with EPI led to a marked increase of N-cadherin protein levels compared to the untreated control cells (Figure 2.8.A); N-cadherin up regulation was attenuated by combination with Docetaxel. By 24hrs-post treatment, the key phenotypic change of elevated N-cadherin associated with EMT induction was restored to control levels (Figure 2.8.A). No significant changes in levels of Twist, E-cadherin or N-cadherin temporal expression were detected. A modest decrease in the cytoskeleton protein, cofilin was evident after 24hrs of DOC or EPI monotherapy.

The EMT landscape was interrogated in the *in vivo* setting of 22Rv1 prostate tumor xenografts (from Figure 2.1.B). E-cadherin expression was increased in response to both Docetaxel and EPI given as single agents, while N-cadherin levels were decreased compared to the untreated controls (Figure 2.8.B) consistent with the phenotypic reversion of EMT to MET in prostate tumor epithelial cells. Interestingly, the combination treatment led to an apparent decrease in E-cadherin and upregulation of N-cadherin protein levels pointing to EMT induction. Tissue levels of vimentin were increased after treatment with EPI or Docetaxel given as single agents, an effect that was blocked by the combination treatment (Figure. 2.8.B).

The temporal changes in gene expression for EMT regulators were interrogated by analysis of mRNA expression levels by qRT-PCR. The results summarized in Figure 2.8 indicate that consistent with EPI increasing levels of N-cadherin protein at 6hrs, levels of N-cadherin mRNA were also elevated within the same period of exposure to EPI, compared to levels observed for CSS control (Figure 2.8.C). Upregulation of mRNA for vimentin and E-cadherin was also detected within 6hrs of treatment with EPI. After longer treatment periods (12, 24 and 24hrs), the levels for both proteins were restored to levels comparable to the controls. A significant increase in N-cadherin mRNA was

detected in response to Docetaxel (as single agent) at 1 and 3hrs, but with no effect on vimentin mRNA (Figure 2.8.C). The combination treatment of EPI and Docetaxel for 24hrs resulted in a significant increase in the levels of mRNA for both N-cadherin and vimentin. A significant induction of Snail mRNA was observed in response to both monotherapies and this was further enhanced in response to the combination of Docetaxel with EPI (Figure 2.8.C). Within the first hour of combination treatment, there was a significant induction in Twist gene expression (Figure 2.8.C). Elevated expression of N-cadherin occurs in response to attenuated AR transcriptional activity or reduced levels of expression of AR (Jennbacken et al., 2010; Tanaka et al., 2010). Thus, these findings support the "programming" of EMT by the AR N-terminal inhibitor, to potentially drive the sensitivity/resistance of CRPC 22Rv1 cells to microtubule-targeting taxane-based chemotherapy (Figure 2.8.D).

Conclusions

Taxane-based chemotherapy is an effective treatment for castration-resistant-prostate cancer (CRPC) via stabilization of microtubules. Previous studies identified that the inhibitory effect of microtubule targeting chemotherapy on androgen receptor (AR) activity was conferred by interfering with AR intracellular trafficking. The N-terminal domain (NTD) of AR was identified as a tubulin-interacting domain that can be effectively targeted by the novel small molecule inhibitor, EPI. Taken together this evidence provided the rationale that targeting AR nuclear translocation and activity via a combination of an antagonist of the AR NTD and taxane-based chemotherapy may enhance the therapeutic response in CRPC. The present study investigated the anti-tumor efficacy of a combination of EPI with Docetaxel chemotherapy, in cell models of

CRPC, harboring the AR splice variants in addition to the full length AR. My studies demonstrate that there was no significant effect on the androgen-mediated nuclear transport of AR variants and AR transcriptional activity by Docetaxel. The therapeutic response to Docetaxel was enhanced by inhibition of the NTD of AR (by EPI) through cycling of EMT to MET among prostate cancer epithelial cells. These results support that transient "programming" of EMT by the AR NTD inhibitor, potentially drives the sensitivity of prostate tumors with differential distribution of AR variants to microtubule-targeting chemotherapy.

Therapeutic response durations to androgen-depletion in advanced metastatic prostate cancer are variable and prostate tumors nearly always become resistant and ultimately lethal. Diverse mechanisms have been implicated in driving aberrant AR function, including intra-tumoral synthesis of androgens from inactive precursors, increased AR expression, truncated AR with constitutive activity, ligand-independent activation of AR signaling and alterations in nuclear receptor co-activators (Debes & Tindall, 2004a; Knudsen & Penning, 2010). Novel pharmacologic targeting of these mechanisms has provided some validation in the clinical setting in the treatment of CRPC, such as those that require the AR ligand-binding domain (Lunardi et al., 2013). A new paradigm has emerged in therapeutic targeting of microtubule-mediated androgen signaling in prostate cancer, as growing evidence indicates that taxane–induced stabilization of microtubules inhibits the nuclear translocation of the androgen-AR complex and impairs AR transcriptional activation (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; Gan et al., 2009a; M.-L. Zhu et al., 2010a). My findings, as presented in this chapter, indicate that Docetaxel treatment causes only a modest inhibition of the AR nuclear localization, although there was a significant suppression of AR-transcriptionally activated gene expression, in the 22Rv1 CRPC prostate cancer

cells. Considering that these cells express a mixture of the full-length AR as well as the AR variants, this was not entirely unexpected. These results gain mechanistic support from recent evidence by Chan and colleagues demonstrating that AR splice variants activate AR target genes and promote prostate cancer growth independent of canonical AR nuclear localization signal (S.C. Chan et al., 2012). More recently it was reported that the nuclear accumulation and transcriptional activity of AR V7 variant was not affected by microtubule-targeting chemotherapy (Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, Plymate, et al., 2014), in full support of this work. Thus one may argue that while nuclear AR localization is functionally critical for controlling AR (full length) transcriptional activation of target genes in androgen-dependent prostate cancer, other distinct pathways may exist to regulate the AR variants nuclear translocation in CRPC, thus driving therapeutic resistance to microtubule-targeting chemotherapy and/or anti-androgens. Interestingly enough, the AR V7 lacks the hinge region which facilitates microtubule targeting and navigates AR nuclear trafficking (Tanner et al., 2010; Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, Plymate, et al., 2014). Our findings on the modest effect of microtubule-targeting treatment on AR V7 transcriptional activity are in accord with the above mechanistic evidence.

My observations indicate that the microtubule motor protein, dynein can efficiently facilitate the cytoplasmic AR trafficking along the microtubules and its nuclear translocation in the presence of androgens, resonates with previous reports (Darshan et al., 2011). In addition, I found that the status of the AR dictates the cellular response to Docetaxel treatment (alone or in combination with EPI) in terms of changes in dynein localization causing punctate sequestration in nuclear and cytoplasmic fractions in CRPC 22RV1 cells (variant AR), while in the androgen-sensitive LNCaP cells (full length AR), treatment leads to massive distribution of dynein throughout the cytosol, promoting

the nuclear export of AR (Figure. 2.8.D). Mechanistically, a differential association with microtubules and the dynein motor protein has been shown for the clinically significant splice variants ARv567es and AR V7, with the AR V7 unable to interact with dynein and ultimately not having an effect on AR nuclear transport and transcriptional activity (Thadani-Mulero et al., 2014). This evidence together with our present results, suggest that dynein may be engaging additional cytoplasmic partners such as HSP90, as well as cytoskeleton modulators to generate a new dynamic of interactions with full length AR versus AR variants, effective targeting of which can potentially bypass cross-resistance to Docetaxel and anti-androgens in advanced prostate cancer (Figure 1.6).

My studies have shown for the first time that combination of the microtubule-targeting agent, Docetaxel with targeting the AR NTD with EPI leads to enhanced anti-tumor action possibly via changes in the EMT landscape and the cytoskeleton of prostate cancer cells harboring AR variants. A functional significance of the EMT process in therapeutic response to microtubule-targeting is supported by the recently reported association between reduced E-cadherin expression and Docetaxel-resistance in prostate cancer cells (Puhr et al., 2012). Reduced E-cadherin expression promotes loss of cell adhesion, cell polarization, and gain of cell migration, which leads to invasion and metastases (J. Yang & R. A. Weinberg, 2008; Yilmaz & Christophori, 2009). Moreover there is growing clinical evidence implicating EMT as a cellular mechanism conferring therapeutic resistance, development of metastases, and contributing to patient mortality (Puhr et al., 2012). Considering that functional disruption of the androgen/AR signaling axis is associated with EMT induction (Matuszak & Kyprianou, 2011b; Y. Sun et al., 2011; M. Zhu & Kyprianou, 2010), Docetaxel administration prior to androgen deprivation may result in improved therapeutic outcomes via navigating the EMT-MET cycling, towards cytoskeleton remodeling and sensitizing prostate tumor cells

to androgen- depletion mediated apoptosis. An alternative mechanistic scenario is that mutations in tubulin may affect the binding sites of taxanes leading to therapeutic resistance. One may argue that when taxanes are structurally unable to effectively bind tubulin, the microtubules can no longer become stabilized, an effect that fails to elicit disruption of cell cycle progression, and consequentially the AR is free for a nuclear translocation (schematically illustrated on Figure. 2.8.D). Treatment of CRPC with EPI, an AR NTD inhibitor, leads to decreased transcriptional activity of both full-length AR and constitutively active truncated variants (Andersen et al., 2010; Myung et al., 2013) and is thereby an attractive target for prohibiting AR from the nucleus to remain in the cytoplasm using taxanes as a combination therapy.

In summary, the present pre-clinical studies demonstrate that the combination strategy of targeting tubulin (by taxane chemotherapy) -and the NTD of AR variants association (by EPI) and cellular localization can impair CRPC by reversing EMT and navigating EMT-MET cycling. These findings provide a new insight into a potential mechanism for overcoming cross-resistance driven by differential status, and activity of AR, depending on its nuclear/cytosolic localization.

Figure 2.1: Combination of an AR NTD Inhibitor and Docetaxel Impairs Growth of Castration Resistant Prostate Cancer *in vitro* and *in vivo*. Panel A, the human prostate cancer cells 22Rv1 were treated with EPI (25µM), or Docetaxel (DOC) (1µM), as single agents or in combination (DOC+EPI), for 24hrs and cell death was assessed on the basis of MTT assay after exposure the drugs. Values represent the average of three experiments (in triplicates) ±SEM. Statistical significance was set at $p<0.05$ (* compared to CSS, ** compared to DOC).

A.

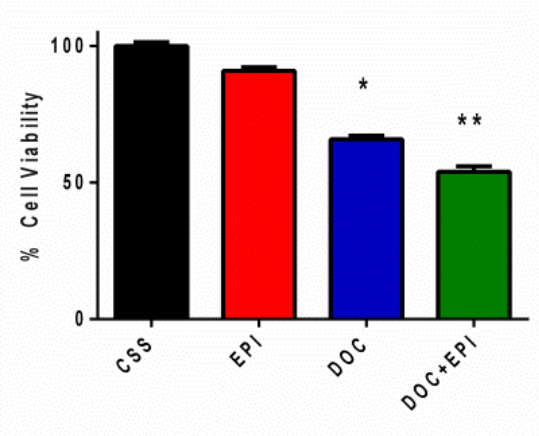


Figure 2.1 continued, Combination of an AR NTD Inhibitor and Docetaxel Impairs Growth of Castration Resistant Prostate Cancer *in vitro* and *in vivo*. Panel B, *In vivo* anti-tumor action of Docetaxel (microtubule-targeting chemotherapy) and EPI (targeting of the N-terminal domain of AR) in 22Rv1 human prostate cancer xenografts. Palpable tumor-bearing mice were treated with CMC (control), EPI alone (200mg/kg), Docetaxel alone (DOC) (15mg/kg), or the combination of the two agents (EPI +DOC) and tumor measurements were conducted as described in "Approach" (n=5/treatment group). Tumor xenografts were surgically excised 2 days after the last treatment (11 days). Error bars represent SEM. ** shows statistically significant difference at $p<0.05$.

B.

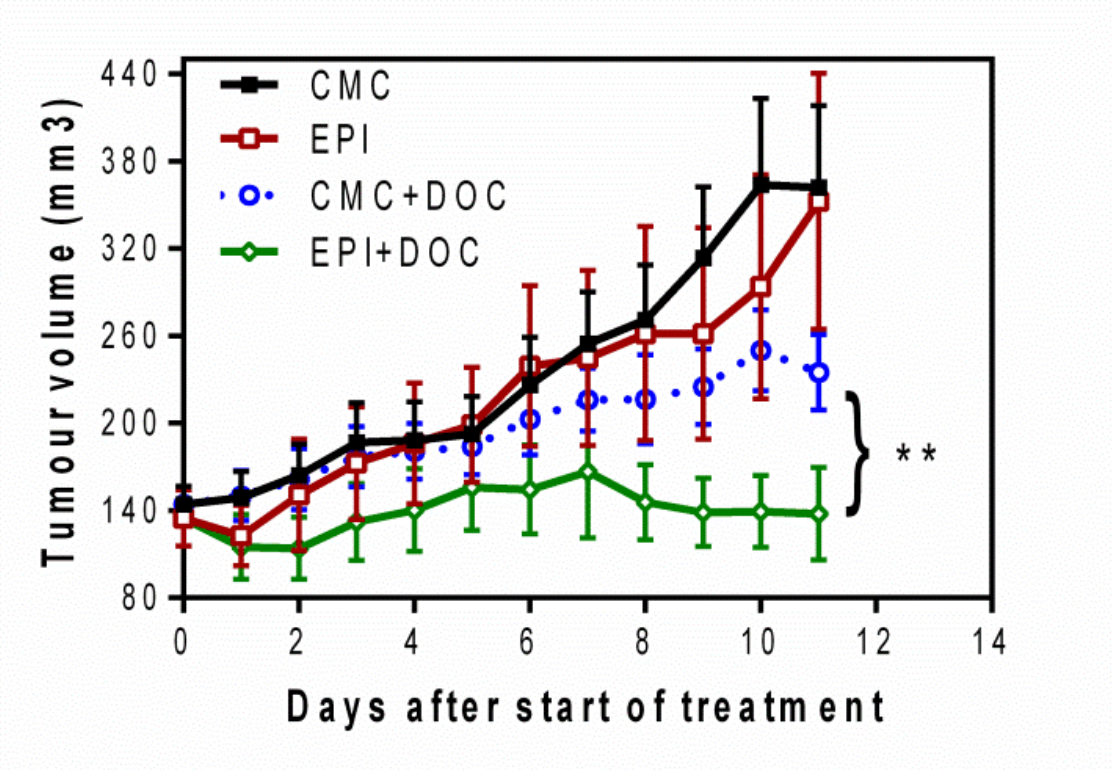
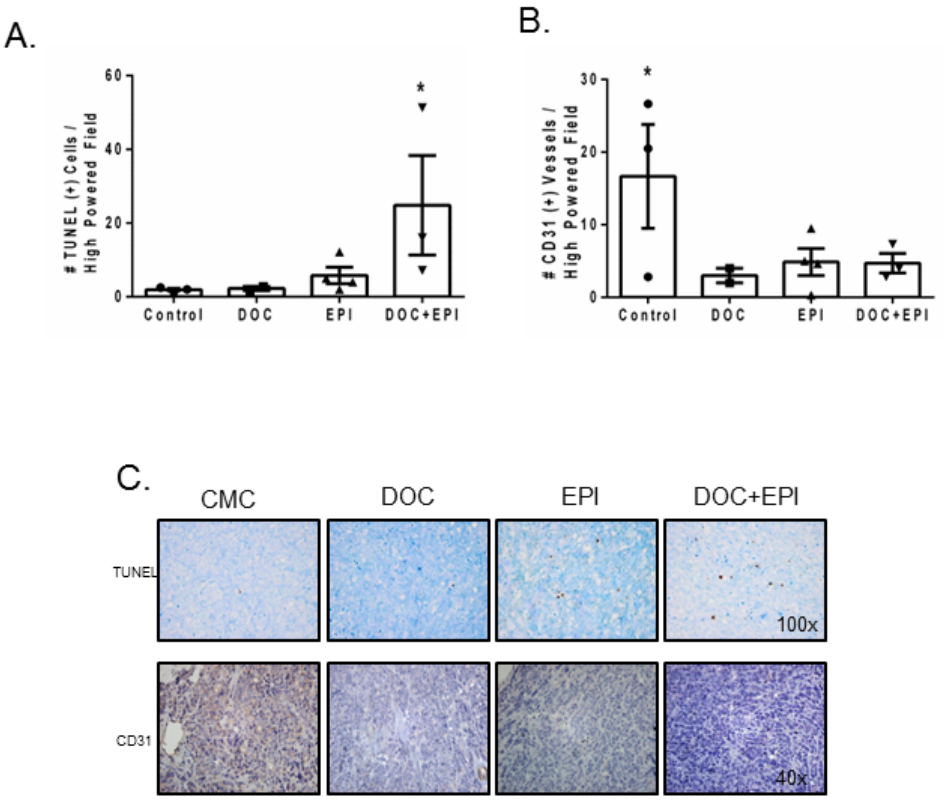


Figure 2.2: Effect of DOC and EPI Combination on Apoptosis and Vascularity of CRPC Xenografts. Panel A, Immunohistochemical quantitation of apoptosis via TUNEL assay using serial sections of prostate tumor 22Rv1 xenografts. Number of TUNEL positive cells per high powered field counted in three separate, non-continuous fields by two independent reviewers (N.K., S.K.M.) Panel B reveals the numerical quantification of CD31 positivity in sections of prostate tumor 22Rv1 xenografts to assess the consequences of various treatments on tumor vascularity. Number of CD31 positive vessels per high powered field counted in three separate, non-continuous fields by two independent reviewers (N.K., S.K.M.). Panel C**,** Detection of apoptosis by TUNEL assay in 22Rv1 human prostate cancer xenograft sections from untreated control (CMC) and treated tumor-bearing mice (DOC, EPI or the combination treatment). Serial sections were subjected to CD31 immunoreactivity to assess tumor vascularity. Magnification 200X.



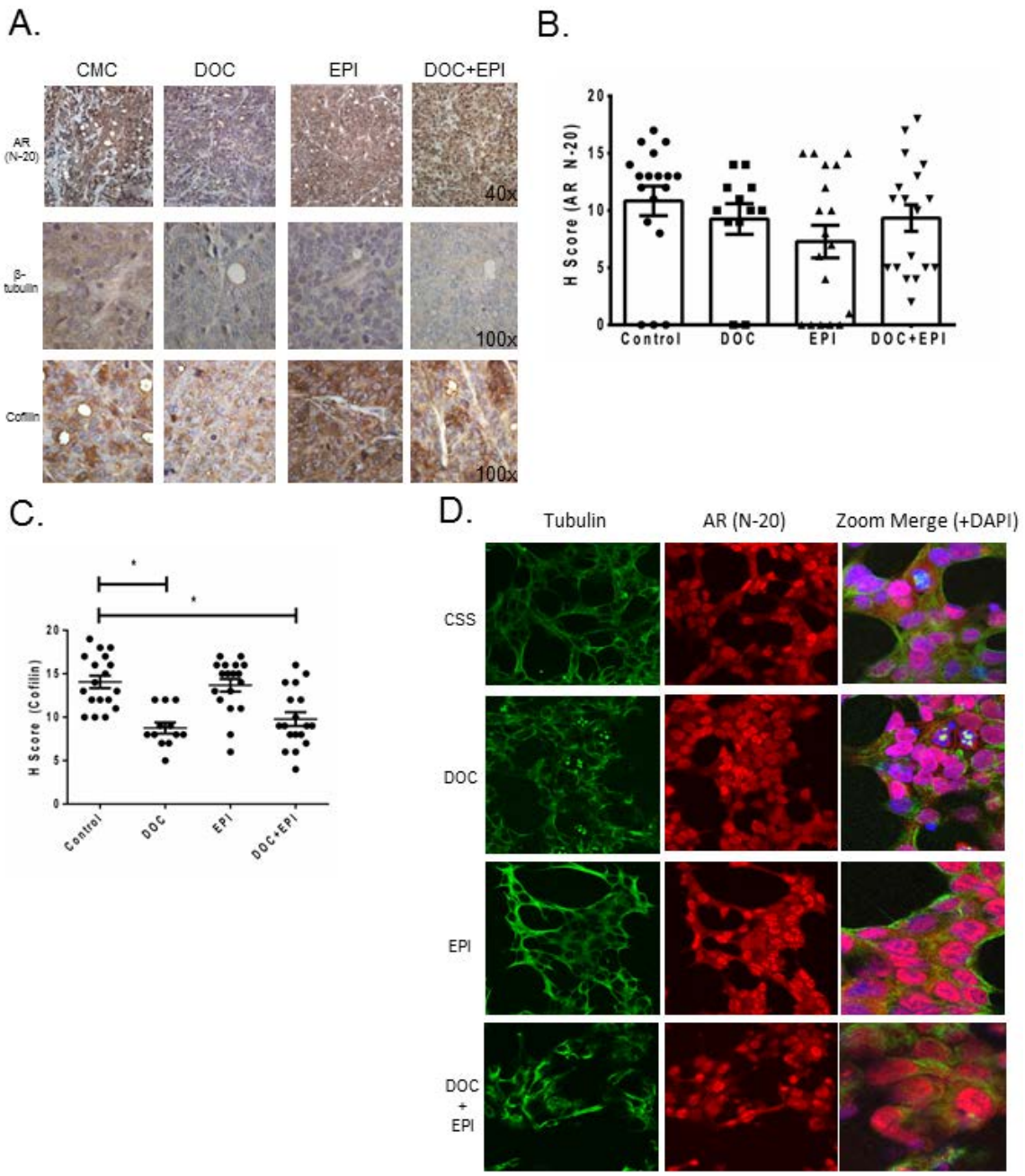

A.
CMC
DOC
EPI
DOC+EPI
AR (N-20)
β-tubulin
Cofilin
40x
100x
100x
B.
H Score (AR N-20)
Control
DOC
EPI
DOC+EPI
C.
H Score (Cofilin)
Control
DOC
EPI
DOC+EPI
D.
Tubulin
AR (N-20)
Zoom Merge (+DAPI)
CSS
DOC
EPI
DOC + EPI

Figure 2.3: Effect of Microtubule and NTD AR Targeting on AR and Cytoskeleton in CRPC. Panel A, To determine the effect of treatment on the expression and cellular localization of the specific proteins, immno-histochemical profile of AR (N-20), β-tubulin (microtubules) and cofilin (actin cytoskeleton) as evaluated in paraffin-embedded serial sections from 22Rv1 prostate tumor xenografts. Magnification 40X and 100X as indicated. Panel B, numerical quantification of total AR expression in 22Rv1 xenograft sections before and after the various treatments (based on H-scoring). H-score calculation: on a scale of 1-10, value for intensity of staining and value for percentage of cells in field positive for staining assigned and averaged based on observation of three separate, non-continuous fields of specimen. Panel C, quantitative results of cofilin immunoreactivity in 22Rv1 xenograft sections (representative images shown on panel A). Docetaxel alone, or in combination with EPI led to a significant decrease in cofilin ($P<0.05$). Quantification represents the average (mean) of three fields, evaluated by two independent reviewers ± SEM. Statistical significance determined by one-way ANOVA; * indicates $P<0.05$. Panel D, reveals the confocal, immunofluorescent images of tubulin (green) and AR (N-20) (red) localization and expression in 22Rv1 cells before (CSS) and after treatment with DOC, EPI, or combination (4hrs).

Figure 2.3 continued, Effect of Microtubule and NTD AR Targeting on AR and Cytoskeleton in CRPC. Panel E, confocal immunofluorescent microscopy analysis of 22Rv1 cells treated with EPI, DOC, as single agents or a combination of EPI+DOC for 6hrs, in the presence of DHT (1nM).

E.

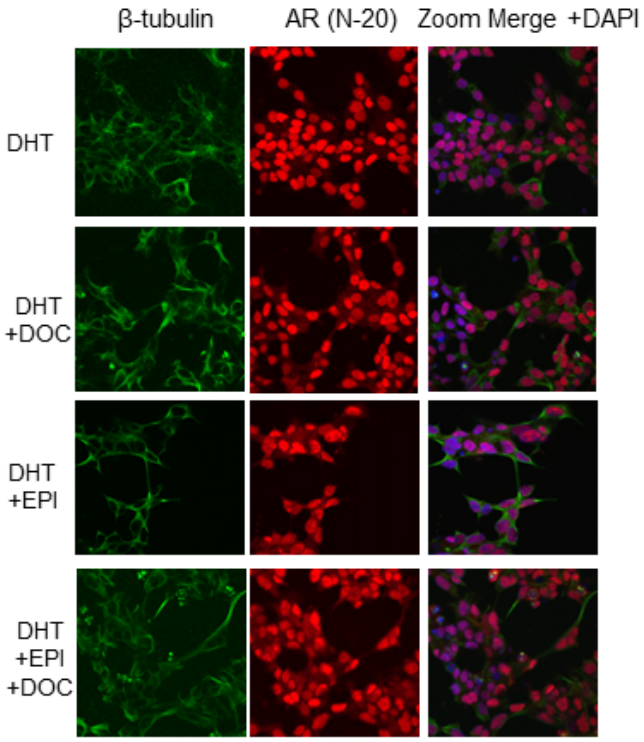
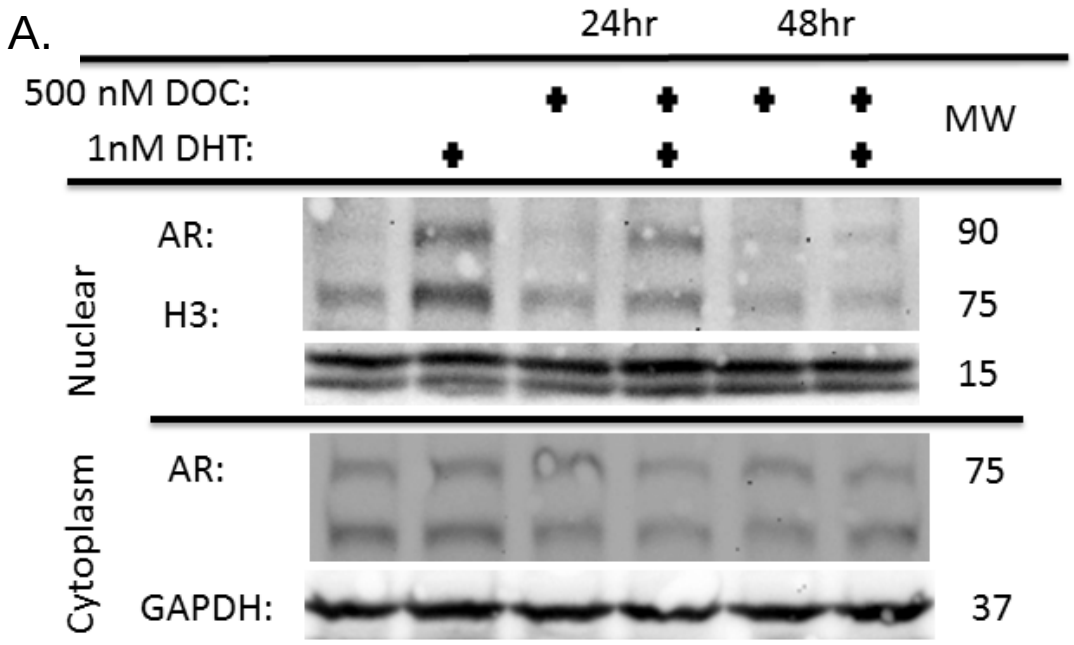


Figure 2.4. Impact of Androgens on the Effect of Taxane Monotherapy on AR Localization. Panel A. 22Rv1 cells treated with 500nM DOC for 24 and 48hrs in presence and absence of 1nM DHT. Subcellular fractionation analysis indicating that DOC decreases full length nuclear AR and ARv7, but does not effect on AR or V7 variant in in cytoplasm of 22Rv1 cells.



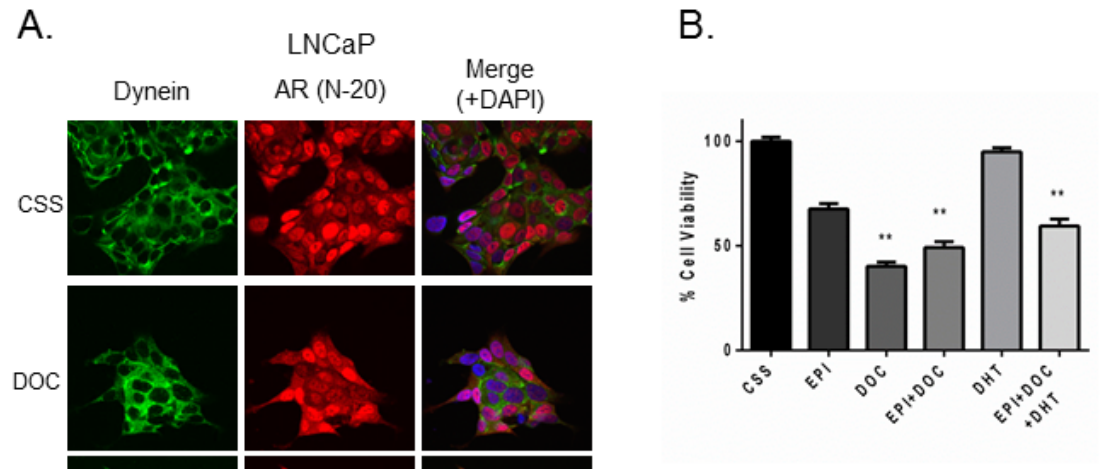

A.
LNCaP
Dynein
AR (N-20)
Merge
(+DAPI)
CSS
DOC
EPI
EPI
+DOC
DHT
DHT
+DOC
DHT
+EPI
DHT
+EPI
+DOC
B.
% Cell Viability
100
50
0
**
**
**
CSS
EPI
DOC
EPI+DOC
DHT
EPI+DOC
+DHT

Figure 2.5: Cell Death in Response to Microtubule Targeting (Taxane), N-terminal Targeting of AR or the Combination in the Androgen-Sensitive Human Prostate Cancer Cells, LNCaP. Panel A. Reveals the characteristic images of confocal microscopy analysis of dynein cellular distribution and AR nuclear expression/localization in LNCaP cells after the various treatments. LNCaP were treated with DOC, EPI as single agents or in combination (6hrs) in the presence of DHT. 40X Oil Objective, 400X magnification. Panel B. Cell viability was assessed using the MTT assay in LNCaP cells (and expressed as a percentage relative to CSS control medium). Cells treated with Docetaxel and EPI combination in the presence or absence of androgens (DHT). Values are the mean of three independent experiments performed in triplicate +/- SEM. Statistical significance at **P<0.05 (Two-tailed, unpaired, Student's t-test) compared to CSS treated controls.

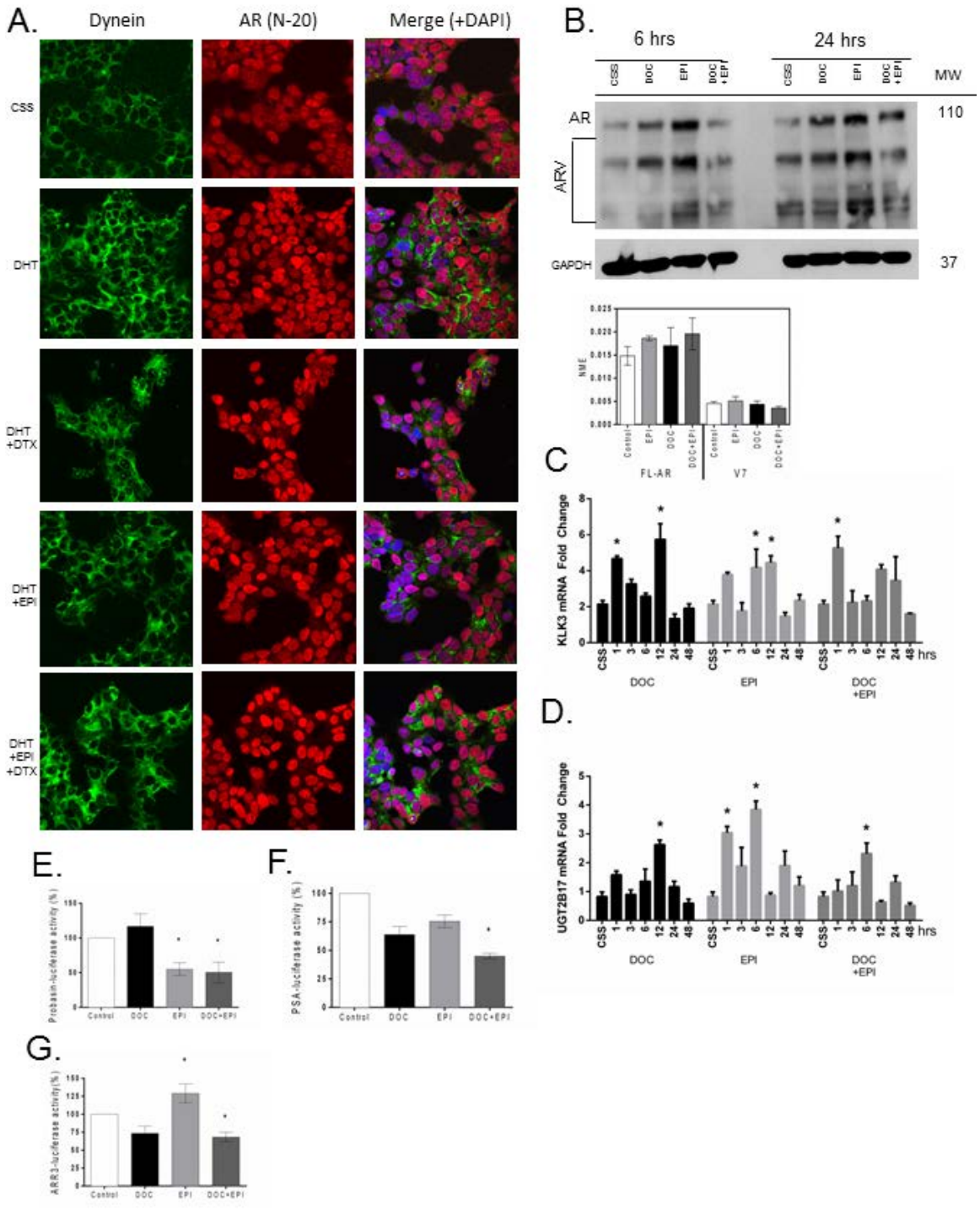

A.
Dynein
AR (N-20)
Merge (+DAPI)
CSS
DHT
DHT +DTX
DHT +EPI
DHT +EPI +DTX
B.
6 hrs
24 hrs
CSS
DOC
EPI
DOC +EPI
MW
AR
ARv
110
GAPDH
37
NME
Control
EPI
DOC
DOC+EPI
FL-AR
V7
C
KLK3 mRNA Fold Change
hrs
DOC
EPI
DOC +EPI
D.
UGT2B17 mRNA Fold Change
E.
Probasin-luciferase activity (%)
F.
PSA-luciferase activity (%)
G.
ARR3-luciferase activity (%)

Figure 2.6. Effect of Targeting Microtubules and N-terminal AR on Cytosolic Protein Association and Nuclear Localization /Activity in 22Rv1 Prostate Cancer Cells. Panel A, Confocal microscopy of dynein IC ½ and AR (N-20) expression and localization in 22Rv1 prostate cancer cells (+DAPI). 22Rv1 cells were exposed to DOC, EPI (as single agents), or in combination (4hrs) and were then treated with DHT (1nM) for 1hr. Representative images shown, 40X oil objective, 400X magnification. Panel B, Western blot analysis of AR protein expression profile in 22Rv1 cells after treatment with DOC, EPI or combination for 6 and 24hrs. Expression normalized to GAPDH as loading control. MW of the full length AR and the variants are shown to left and densitometry of full length AR indicated below blot and densitometry of protein bands on Western blot for expression of AR. Panels C and D, RT-PCR analysis of PSA/KLK3 mRNA expression (FL-AR target gene) and UGT2B17 mRNA expression (AR V7 target gene) respectively in 22Rv1 human prostate cancer cells in response to indicated treatments. Numerical values represent the mean of three independent experiments measured in duplicate +/- SEM; (* indicated $P<0.05$). Panels E, F and G represent transcriptional reporter assays in 22Rv1 cells. Cells were transiently transfected with PB-luciferase (E), PSA (6.1kb)-luciferase (F), or ARR3-Luc reporters (G) and pre-treated with EPI, DOC, or combination (EPI+DOC) prior to incubation with R1881. Luciferase activity is represented as percentage of vehicle control activity in response to androgen. Bars represent SEM of three independent experiments. Luciferase reporter assays performed by lab of Marianne Sadar (Vancouver Genome Center).

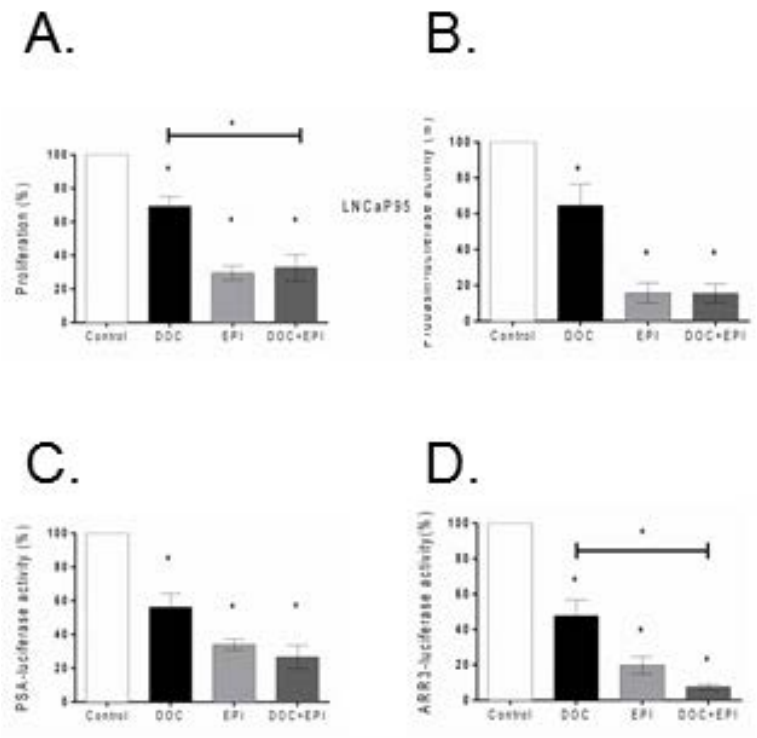

A.
B.
LNCaP95
Proliferation (%)
Control
DOC
EPI
DOC+EPI
C.
D.
PSA-luciferase activity (%)
ARR3-luciferase activity(%)
E.
control
EPI
DOC
EPI+DOC

Figure 2.7: Effect of Combined Targeting of Microtubules and AR NTD in Androgen-Sensitive LNCaP95 Cell Growth and AR Activity. Panel A, Cell proliferation assay in LNCaP95 prostate cancer cells, that harbor the FL AR as well as the AR V7, pre-treated with EPI, DOC or combination for 24hrs prior to measurement of BrdU incorporation. Bars represent the mean ± SEM of three independent experiments. Panels B, C and D represent analysis of transcriptional activity assays in LNCaP95 cells. Cells were transiently transfected with PB-luciferase (B), PSA (6.1kb)-luciferase (C), or ARR3-Luc reporters (D) and pre-treated with EPI, DOC, or combination prior to incubation with R1881. Luciferase activity is represented as percentage of vehicle control activity in response to androgen. Bars represent the mean ± SEM of at least three independent experiments. *P<0.005 (Two-tailed, unpaired, Student's t-test). Panel E, morphological appearance of the LNCaP95 cells (as detected by light microscopy), after the various treatments.

Figure 2.8: Impact of AR Variant on EMT in CRPC 22Rv1 Cells in Response to Combination Targeting of the AR NTD and Microtubules. Panel A. Western blot analysis of critical EMT regulator proteins after 6 and 24hrs of treatment of 22Rv1 cells with DOC, EPI or combination. Expression levels for E-cadherin, N-cadherin, β-catenin, Twist and cofilin are shown. GAPDH was used as a loading control. The MW of individual proteins is shown (kDa).

A.

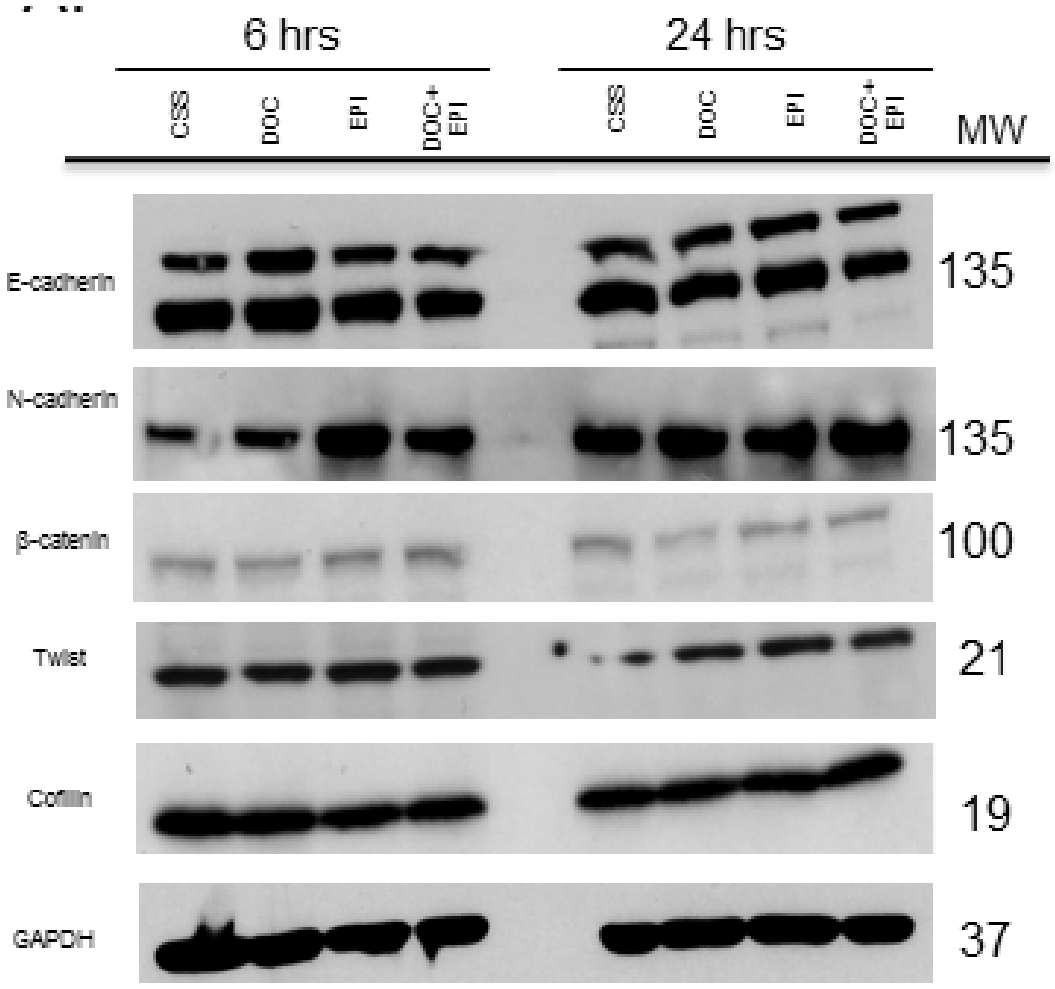


Figure 2.8 continued: Impact of AR Variant on EMT in CRPC 22Rv1 Cells in Response to Combination Targeting of the AR NTD and Microtubules. Panel A continued. EMT and Cytoskeleton Effector Profiling in Response to Treatment. Densitometric analysis of the protein expression bands from Western blot analysis of EMT and cytoskeleton regulator proteins. E-Cadherin, N-Cadherin, β-catenin, Twist, Cofilin.

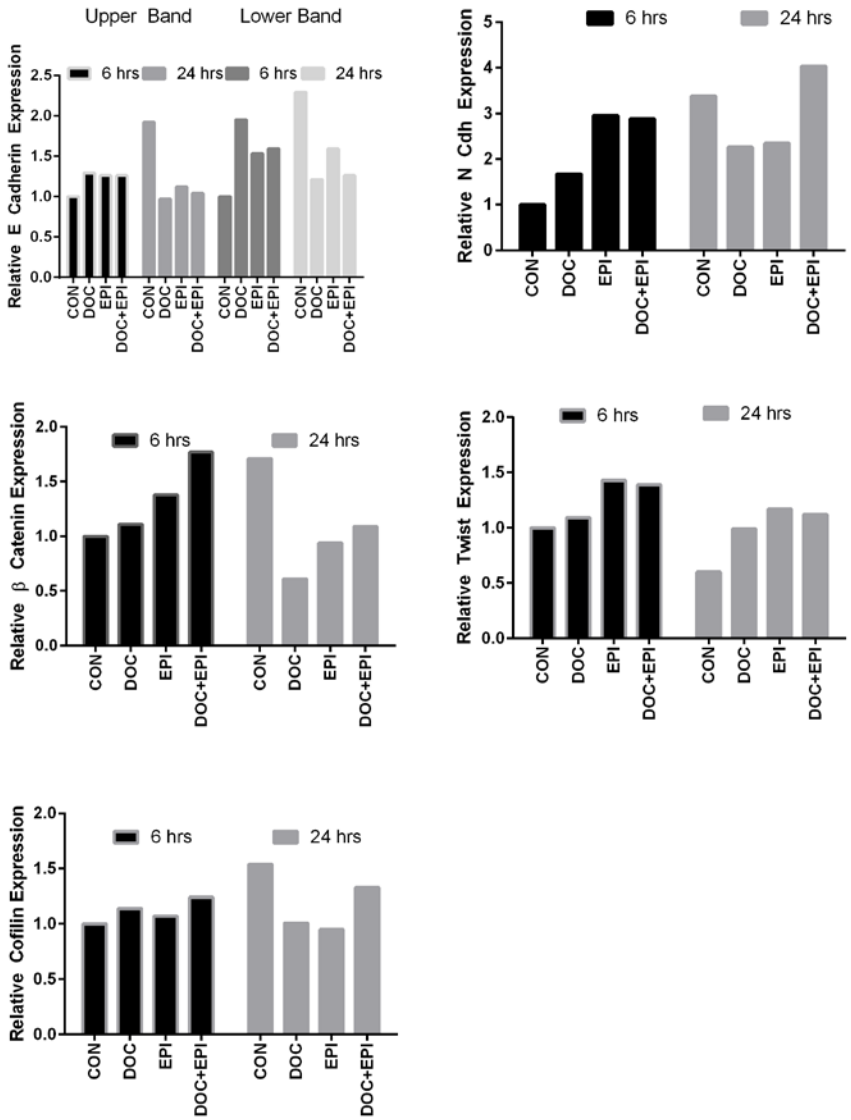


Figure 2.8 continued, Impact of AR Variant on EMT in CRPC 22Rv1 Cells in Response to Combination Targeting of the AR NTD and Microtubules. Panel B, Serial sections of 22Rv1 prostate tumor xenografts from untreated control (CMC) and treated tumor-bearing mice (DOC, EPI, or combination, obtained as in Fig.2.1 B), were subjected to immunohistochemical analysis for the EMT markers E-cadherin, N-cadherin and vimentin. Magnification 100X.

B.

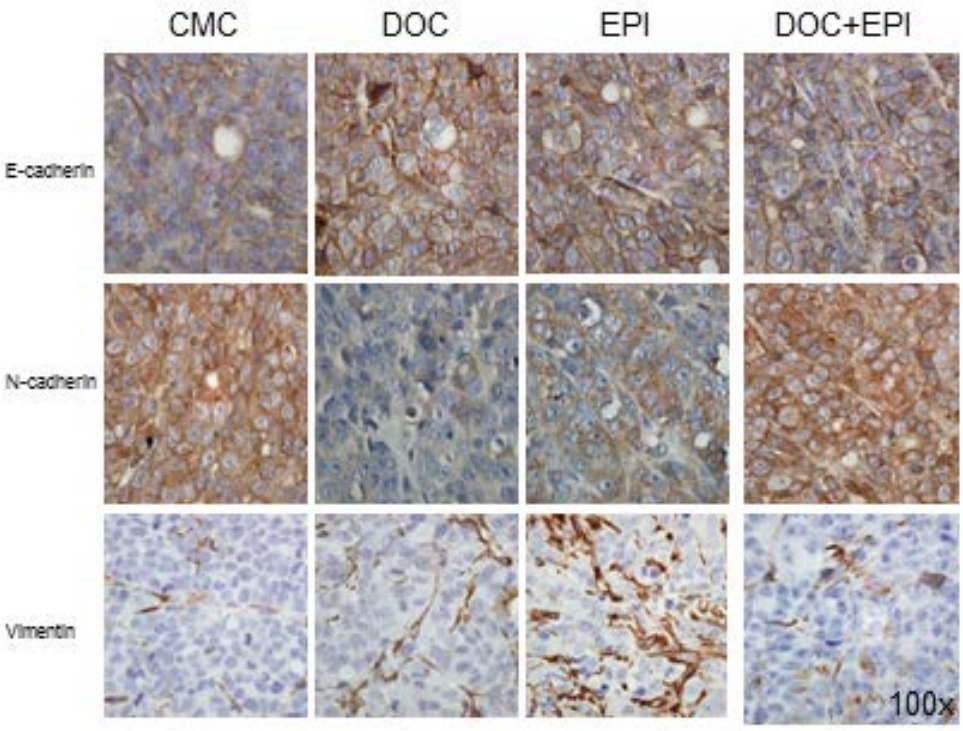


Figure 2.8 continued, Impact of AR Variant on EMT in CRPC 22Rv1 Cells in Response to Combination Targeting of the AR NTD and Microtubules. Panel C. Reveals the temporal analysis of gene expression of EMT regulators. 22Rv1 prostate cancer cells were treated with DOC, EPI or the combination (1, 3, 6, 12, 24 and 48hrs) and mRNA expression was analyzed by RT-PCR. The gene expression profile of mRNA for the EMT effectors, E-cadherin (CDH1), N-cadherin (CDH2), and vimentin (VIM), as well as for the transcriptional regulators SNAIL1 and Twist, in response to treatments is shown. Values represent the mean +/- SEM of duplicate measurements from three independent experiments; (*) denotes statistical significance at $p<0.05$.

C.

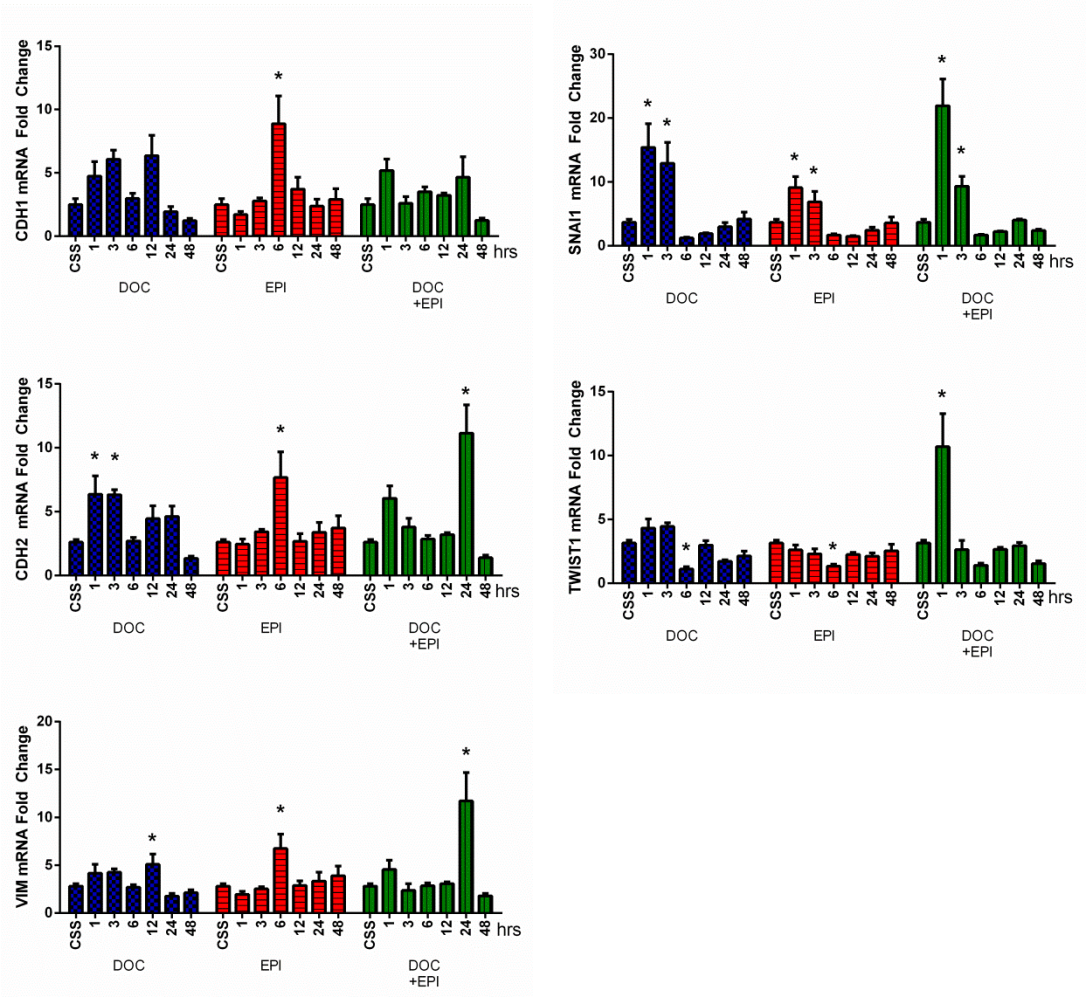
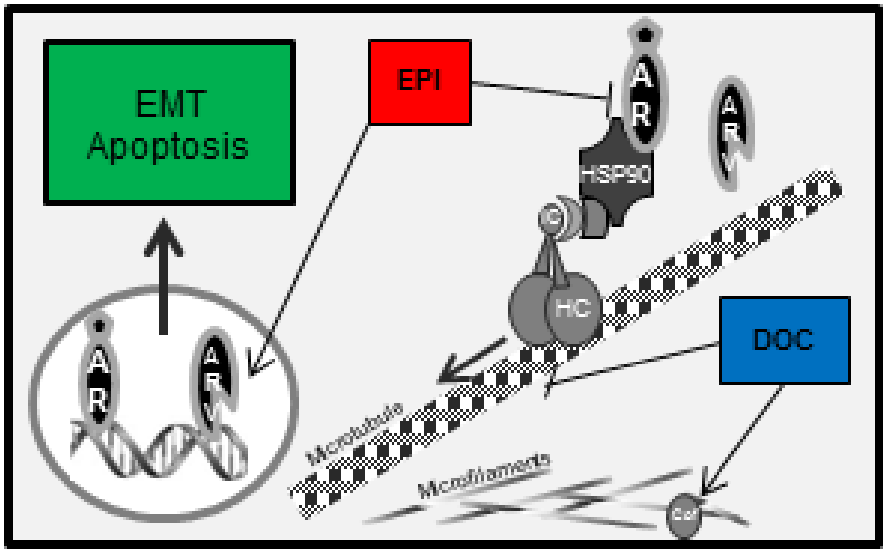


Figure 2.8 continued, Impact of AR Variant on EMT in CRPC 22Rv1 Cells in Response to Combination Targeting of the AR NTD and Microtubules. Panel D, Schematic Diagram projecting the potential interactions of AR with cytoplasmic proteins (dynein, tubulin, cofilin) that may control its nuclear translocation and transcriptional activity of target genes, mediating EMT and apoptosis in CRCP cells.

D.



Chapter 3. Cabazitaxel Induces Mesenchymal Epithelial Transition (MET) and Overcomes Phenotypic Resistance in Advanced Prostate Cancer

Background and Significance

As described in Chapter I, combination of taxane-microtubule targeting chemotherapy and anti-androgen therapies have demonstrated additional survival benefits in late stage CRPC patients. Previous studies from our lab and others demonstrated that Docetaxel chemotherapy inhibits AR activity by blocking its nuclear translocation (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M. Zhu & Kyprianou, 2010). The present study pursued the anti-tumor efficacy of a combination strategy of the AR antagonist Enzalutamide, MDV3100 (MDV), with second line taxane chemotherapy, Cabazitaxel (CBZ) *in vitro* and *in vivo* models of advanced CRPC.

Progression to metastatic castration resistant prostate cancer (mCRPC) is characterized by aberrant over expression of androgen receptor (AR), *de novo* intra-prostatic androgen production, and cross talk between androgen signaling with other oncogenic pathways (Kahn et al., 2014; Mohler, 2008). Anti-androgen therapies Abiraterone Acetate and Enzalutamide (next generation anti-androgens) effectively target the androgen signaling axis (Acloque et al., 2009; Beer et al., 2014; Ryan et al., 2013) however, due to the addiction of CRPC cells to AR signaling and its splice variants resistance develops driving CRPC progression (Antonarakis & Eisenberger, 2013; C. D. Chen et al., 2004). Taxanes are the mainstay of chemotherapy for patients who developed resistance to anti-androgen strategies and progressed to metastatic CRPC. First generation taxane chemotherapies, Docetaxel (Taxotere©) effectively target the cytoskeleton by stabilizing the interaction between β-tubulin subunits of microtubules preventing de-polymerization, inducing G2M arrest and apoptosis (Huizing et al., 1995).

Un-liganded AR is sequestered in the cytoplasm by the HSP90 super-complex until it encounters its' cognate ligand, dihydrotestosterone (DHT), dimerizes and translocates via microtubules into the nucleus (B.J. Feldman & D. Feldman, 2001; Thadani-Mulero, Nanus, & Giannakakou, 2012). Nuclear AR binds androgen responsive elements of DNA and activates transcription of androgen responsive genes promoting prostate cell growth (B.J. Feldman & D. Feldman, 2001).

Evidence from this laboratory, confirmed by others, established that stabilization of microtubules by Docetaxel chemotherapy inhibits the AR translocation into the nucleus, thus preventing its transcriptional activity (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a; M. Zhu & Kyprianou, 2010). Additionally, taxanes lead to an increase in Forkhead box 01 (FOXO1), a transcriptional repressor of AR, consequently resulting in inhibition of ligand-dependent and ligand-independent transcription (Gan et al., 2009b), as well as down-regulation of AR resulting in decreased PSA expression (Mistry & Oh, 2013). The therapeutic impact of taxanes in impairing metastatic CRPC and improving overall survival in patients with advanced disease, has been attributed to microtubule stabilization and AR targeting ADT (Fitzpatrick & de Wit, 2014; Mistry & Oh, 2013). Despite showing initial efficacy and a proven survival advantage in patients with mCRPC, resistance to Taxane treatment invariably develops leading to disease progression in approximately 7.5 months (Loriot et al., 2013). Mechanisms implicated in the development of Docetaxel resistance, include high affinity of the drug for the P-glycoprotein drug efflux pump, mutational alterations in tubulin expression, overexpression of microtubule associated proteins, and induction of EMT (Fitzpatrick & de Wit, 2014; Loriot et al., 2013; Puhr et al., 2012).

A veritable boom of FDA approvals have brought us such promising agents Jevtana© (Cabazitaxel, CBZ), Xtandi© (Enzalutamide, MDV3100, MDV), and Provenge©

(Sipleucel-T) providing additional survival benefits to patients with advanced therapeutically resistant disease (Bianchini et al., 2014; Fizazi et al., 2012). Cabazitaxel is a second-line taxane chemotherapy, a drug developed by addition of two methyl groups to the structure of Docetaxel to significantly decrease its' affinity for the multi-drug resistance protein and P-Glycoprotein pump and increase cellular retention of the drug (Vrignaud et al., 2013). The novel non-steroidal, anti-androgen enzalutamide (MDV) was rationally designed from the crystal structure of the AR (Acloque et al., 2009; Brasso et al., 2014; Fizazi et al., 2012). As discussed in Chapter 1, MDV blocks androgen signaling with a three-headed mechanism. MDV has been shown to prevent binding of AR with DHT, prevent translocation of AR into the nucleus, and inhibit AR from binding to androgen responsive elements on DNA thus driving its therapeutic impact (Fizazi et al., 2012; Thadani-Mulero et al., 2012). However, recently emerging clinical evidence indicates that the AR splice variant V7 confers therapeutic resistance to MDV in select populations of patients with CRPC (Antonarakis et al., 2014; Y. Li et al., 2013).

In this chapter, I describe the results of my recent efforts to pursue the mechanism of anti-tumor action of Cabazitaxel against prostate cancer cell models harboring AR mutations, splice variants and full length AR. Similar to Docetaxel, Cabazitaxel induces apoptosis and G2M arrest. In contrast to Docetaxel, Cabazitaxel it sustains AR nuclear presence, while it reduces its' expression regardless of androgen presence. In CRPC cell models harboring AR splice variants including the V7 variant, combination treatment of Cabazitaxel as a single agent with anti-androgen resulted in a growth response. My findings established for the first the ability of Cabazitaxel to overcome phenotypic resistance in CRPC and promote glandular formation, by reversing

EMT to MET of prostate tumor cell populations in *in vivo* models of prostate cancer progression.

Approach

Cell Lines and Transfections**.** Human prostate cancer cell lines, the androgen independent cell lines PC3 and DU145, the CRPC cancer cell line 22Rv1, and the androgen sensitive human prostate cancer cell lies LNCaP and VCaP were obtained from American Type Culture Collection (ATCC, Manassas, VA). PC3 AR variant transfectants PC3v7, PCv12 and PC3v567es were generated in this lab using plasmids generously provided by Drs. Plymate (University of Washington) and Luo (Johns Hopkins). TGF-β responsive LNCaP TβRII were generated in this lab and have been previously described (Y. Guo & Kyprianou, 1998, 1999) All cell lines, but the VCaP cells, were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (FBS), 100units/ml penicillin and 100µg/ml streptomycin in a 5% CO2 incubator at 37° C. The VCaP cells were cultured in growth medium with DMEM instead of RPMI1640 (ATCC, Manassas, VA). For experiments on androgen responses cells were seeded in 10% charcoal-stripped serum (CSS) and stimulated for 3hrs by dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO) or R1881 (1nM).

Drugs: Cabazitaxel (Jevtana©) was generously provided by Sanofi Aventis. For the *in vivo* administration, Cabazitaxel was prepared by mixing 1 volume of ethanol stock solution, 1 volume of polysorbate 80, and 18 volumes of 5% glucose in sterile water. Solutions were administered intravenously as a slow bolus. Drug doses were adjusted on the basis of body weight at the beginning of treatment. CBZ stock (500 µM) was prepared in 100% ethanol and stored at -20° C in amber bottles. MDV3100 was

purchased from Selleck Chemicals (Houston, TX). For *in vivo* administration in mice, MDV3100 was prepared in dimethyl-sulphoxide (DMSO), diluted with sterile PBS (75% PBS: 25% CBZ DMSO Solution) and injected intraperitoneally.

Antibodies. The antibody against the AR (N-20) protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against tubulin, N-cadherin, MCAK (KIF2C), HSET, CD31, and phospho-Histone H3 were obtained from AbCam Cell Signaling (Cambridge, UK); antibodies against cleaved Caspase-3, GAPDH, and E-Cadherin proteins were obtained from Cell Signaling Technology (Danvers, MA). The ZEB1 antibody was obtained from Bethyl Laboratories (Montgomery, TX).

Western Blot Analysis. Total cellular protein was extracted from cell lysates by homogenization with RIPA buffer (Cell Signaling Technology, Danvers, MA); subcellular fractionation was performed using NE-PER nuclear-cytoplasmic fraction kit (Thermo Scientific.). Protein samples were loaded into 4%-15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and subjected to electrophoretic analysis and blotting. Membranes were incubated with the specific primary antibody (overnight at 4°C) and were subsequently exposed to relevant secondary antibody. For signal detection, membranes were incubated with the Amersham ECL Plus Western Blotting Detection System (Amersham, GE Healthcare, Buckinghamshire, UK) and auto-radiographed using X-ray film (Denville Scientific, South Plainfield, NJ). Protein expression bands were normalized to GAPDH expression (loading control).

Cell Viability Assay. The effect of the various treatments on prostate cancer cell viability was evaluated using the Thiazolyl Blue Tetrazolium bromide (MTT) assay. Cells were seeded into 24-well plates, grown to 60-75% confluence, and treated with vehicle control (DMSO, Sigma-Aldrich, St. Louis, MO), Cabazitaxel, MDV3100, and combination

in RPMI 1640 with 10% CSS (Charcoal Stripped Serum) for 24hrs (or DMEM for VCaP). Following drug treatment, media was aspirated and cells rinsed with PBS then treated with 250µl/well MTT (1mg/ml) for 30mins at 37°C. After incubation, MTT was aspirated and formazan crystals were solubilized with DMSO. Absorbance was measured at 570nm using µQuant Spectrophotometer (Biotech Instruments Inc., Winooski, VT).

Migration Assays. Cells were seeded in 6-well plates and at 65% to 70% density the cell monolayers were wounded. After 24hrs the number of migrating cells towards center of the wound is counted in three different fields.

Quantitative RT-PCR Analysis. *In vitro* samples: RNA was extracted with the Trizol© reagent (Life Technologies, Grand Island, NY) and RNA samples (1µg) were subjected to reverse transcription using the Reverse Transcription System (Promega, Madison, WI). TaqMan real time reverse transcriptase-PCR (Life Technologies, Grand Island, NY) analysis of the cDNA samples was conducted in an ABI7700 Sequence Detection System (Applied Biosystems, Inc, Branchburg, NJ), using the following specific primers: for Prostate Specific Antigen (KLK3; Hs02576345_m1), AR (Hs00171172_m1), KIF2C (Hs00901710_m1), FOXO1 (Hs01054576_m1), KIFC1 (Hs00954801_m1), E-Cadherin (CDH1; Hs01023894_m1), N-Cadherin (CDH2; Hs00983056_m1), Twist (TWIST1; Hs01675818_s1), Vimentin (VIM; Hs00185584_m1) and 18S rRNA (4319413E) (Applied Biosystems, Life Technologies, Grand Island, NY). For the qRT-PCR experiments, data represent mean values from three independent experiments and each sample were analyzed in duplicate. Numerical data for transcript levels were normalized to 18s rRNA in controls and expressed relative to controls.

Immunofluorescent Confocal Microscopy. Cells were plated ($1x10^5$) on cover glass in 6 well plates. After 24-48hrs, cells were exposed to medium (RPMI 1640 with

10% CSS) in the presence of DHT (1nM), CBZ (35-100nM), MDV3100 (1μM) or in combination of the two agents. Following treatment, cells were fixed in 100% methanol and permeabilized with 0.1% Triton X-100 in sterile phosphate buffer saline (PBS). Fixed cells were incubated overnight with primary antibody specific for AR (N-20), and Tubulin (AbCam Cell Signaling, Cambridge, UK), (at 4°C) with gentle rocking and the appropriate Alexa-Fluor (Life Technologies, Grand Island, NY) fluorescent secondary (1.5hrs, room temperature). Slides were mounted using Vectashield mounting medium with DAPI and were visualized using a FV1000 Confocal Microscope (Markey Cancer Center Core, University of Kentucky).

Flow Cytometric Analysis. The human prostate cancer cells harboring the AR variants PC3v567es and 22Rv1 cells were exposed to various treatments (MDV, CBZ, or combination for 24-96hrs), and subjected to washing with PBS in 0.1% bovine serum albumin. Cells were subsequently fixed with 100% ethanol (-20°C). For cell cycle measurement, the cells were incubated with propidium iodide staining solution with RNase A (10ug/mL) overnight, at 4°C. Samples were analyzed for cell cycle progression using Becton-Dickinson FACSCalibur by the flow cytometry core facility at the University of Kentucky.

In Vivo Tumor Targeting Studies. All animal experiments were performed in accordance with the guidelines approved by the Animal Care and Use Committee of the University of Kentucky. Male nude mice (5-6 weeks old) mice were subcutaneously injected with 22Rv1 cells ($2x10^6$ cells) and after tumors were palpable (approximately 3 weeks post-inoculation), mice were divided into four groups, 5 mice/group: (a) Control group receiving 1% medium /0.1% Tween-20 daily via oral gavage; (b) CBZ treatment group, mice received CBZ (Day 1 and 4: 5 mg/kg, Day 8 and 14: 2.5 mg/kg) via tail vein injection for two weeks (c) Enzalutamide group, mice receiving 30 mg/kg MDV3100 via

intraperitoneal injection for 2-weeks; (d) CBZ and MDV3100 combination group, mice receiving both Cabazitaxel and Enzalutamide for 2wks. Tumors were measured twice a week and the volume was calculated (length x width x 0.5236). Prostate tumor xenografts were harvested at 4 days after the last treatment (2wks of drug exposure) and tissue specimens were subjected to histopathological analysis. Formalin-fixed paraffin embedded sections were subjected to immuno-staining for assessing the expression and cellular localization of AR, mitotic kinesins, EMT, vascularity (CD31), and apoptosis (TUNEL, EMD Millipore, Billerica, MA). TUNEL analysis for detection of apoptotic cells *in situ* was performed as previously described (Pu et al., 2009).

Transgenic Mouse Model of Prostate Cancer Progression. Mice were maintained under environmentally controlled conditions and subject to a 12-h light/dark cycle with food and water ad libitum. Animals TRAMP+/DNTGFβRII+ (Pu et al., 2009) (age 16-18 weeks) were matched with littermates and were treated with either vehicle control (VHC) or highest non-toxic dose (HNTD) of CBZ (Day 0 and 3: 10mg/kg; Day 7 and 11: 5mg/kg) dosed intraperitoneally and harvested on Day 14. TRAMP+/DNTGFβRII+ male mice were castrated and followed by treatment with CBZ (Day 3 and 7: 10mg/kg; Day 11 and 15: 5mg/kg) and harvested on Day 18.

Immunohistochemical Analysis. Tissue specimens from human prostate tumor 22Rv1 xenografts and transgenic mouse prostate tumors were formalin fixed and paraffin-embedded; serial sections (5μ), were subjected to immunohistochemical analysis using antibodies against E-cadherin, N-cadherin, Androgen Receptor (N-20), MCAK, HSET, pH3, ZEB1 and CD31. After blocking nonspecific binding (1.5% NGS in TBS-Triton), sections were incubated with primary antibody (overnight, 4°C) and were subsequently exposed to biotinylated goat anti-rabbit IgG (2hrs, room temperature) and horseradish peroxidase-streptavidin (EMD Millipore, Billerica, MA). Signal/Color

detection was achieved with SigmaFast 3, 3'-Diaminobenzidine tablets (Sigma-Aldrich, St. Louis, MO) and counterstained with haematoxylin. TUNEL analysis for detection of apoptotic cells was performed as previously described (Pu et al., 2009). Images were captured via light microscopy (40x and 100x) using an Olympus BX51 microscope (Olympus America, Center Valley, PA). The intensity and level of immunoreactivity was recorded by two independent observers counting three different fields per section (N.K. and S.K.M.).

Statistical Analysis. Student's t test, one-way, or two-way ANOVA were performed using GraphPad Prism 6 software to determine the statistical significance of difference between means / treatments. All numerical data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at P value < 0.05.

Results

Significance of AR Status in Prostate Cancer Cell Response to Cabazitaxel

The pre-clinical efficacy of Cabazitaxel chemotherapy against prostate cancer was originally demonstrated using the androgen-independent human cancer cell line, DU145 (lacking AR) as a model (Vrignaud et al., 2013). To establish the cellular response of androgen sensitive and CRPC cancer cells to CBZ treatment, a panel of human prostate cancer cell lines with varying AR expression status was used. A dose response analysis of prostate cancer cell viability to increasing concentrations of CBZ (10-500 nM) for 96hrs, demonstrated that DU145 cells were highly sensitive to CBZ treatment consistent with earlier reports (Vrignaud et al., 2013) (Figure 3.1, panel A). A time course analysis of the temporal response to increasing treatment periods to CBZ (100nM) was also conducted (Figure 3.1, panel B). The androgen-independent PC3

cells exhibited a similar sensitivity to CBZ as the DU145 cells (Figure 3.1, panels A and B). In contrast, PC3 cells with forced overexpression of the AR splice variant v567es (PC3v567es) were resistant to CBZ even at very high doses of the drug, compared to parental PC3 cells (Figure 3.1, panel A). The CRPC cell line 22Rv1 harboring a mixture of the clinically relevant AR variants as well as the full length AR, exhibited relative resistance to low doses of CBZ and early treatment periods (24-48hrs), but in response to high doses and for longer treatment periods (over 72hrs), there was significant loss of cell viability (Figure 3.1, panels A and B). The androgen sensitive cells VCaP (full length AR) demonstrated resistance to CBZ even at high concentrations (500nM) and after longer treatment (120hrs) compared to other androgen sensitive cell lines, LNCaP and LNCaPTβRII (Figure 3.1, panels A and B).

To examine the effect of AR targeting inhibition by the antiandrogen (MDV3100, MDV) to sensitize prostate cancer cells to CBZ, human prostate cancer cell lines were treated with CBZ alone (100nM) or in combination with MDV (1-10μM). The PC3v567es cells did not exhibit additional loss of cell viability as a result of MDV treatment compared to CBZ alone (except at supra-physiological concentration of the antiandrogen) (Figure 3.1, panel C). For the androgen-sensitive and CBZ resistant VCaP cells (Figure 3.1, panel A), exposure to increasing concentrations of MDV in combination with CBZ resulted in a significant loss of viability ($P<0.05$) (Figure 3.1, panel C). The CRPC 22Rv1 cells (harboring a mixture of full length AR and AR splice variants) exhibit loss of viability in response to CBZ alone; however combination of the taxane with MDV at high concentrations (10μM) led to an increase in cell viability compared to single CBZ treatment (Figure 3.1, panel C), and compared to untreated control cells. Cell cycle analysis revealed that for the PC3v567es cells CBZ treatment promotes G2 and S phase arrest (Figure 3.1, panels D and E). For CRPC 22Rv1 cells, exposure to CBZ alone for

96hrs resulted in a significant G2 arrest that was further increased by the antiandrogen (MDV) treatment (Figure 3.1, panels F and G). Accumulation in S phase was also induced in 22Rv1 cells at 96hr when treated with CBZ alone or in combination with MDV. Treatment of PC3v567es and 22Rv1 cells with CBZ in presence or absence of androgens increases the total cell population in the S and G2 phase.

Recent work from this laboratory demonstrated that combination therapy of taxanes (Docetaxel) and N-terminal targeting of AR with novel anti-androgens enhanced the therapeutic efficacy of taxane against CRPC tumor growth (Martin et al., 2014). Thus, we comparatively analyzed the dose response of prostate cancer cells to Docetaxel (microtubule targeting), MDV (AR targeting), given as single agents or in combination (Figure 3.2, Panel A, B, C). The PC3, PC3v567es, 22Rv1 and LNCaP cells exhibited resistance to MDV but all the cell lines showed partial sensitivity to Docetaxel (Figure 3.2, panels A and B, respectively). Combination of Docetaxel and MDV led to a significant loss of cell viability for all of the prostate cancer cell lines (Figure 3.2, panel C). Transfection-mediated overexpression of clinically relevant AR variants in PC3 cells resulted in stable clones PC3567es, PC3v7 and PCv12 that all exhibited a significant increase in their migration potential compared to parental control cells (Figure 3.2, panel D).

Effect of Cabazitaxel on AR Expression, Localization and Activity

Docetaxel chemotherapy has been shown to impair prostate cancer growth by preventing the physical translocation of cytoplasmic AR into the nucleus ultimately inhibiting the activity of AR-regulated target genes (PSA) (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a; M. Zhu & Kyprianou, 2010). LNCaP and VCaP cells were grown in CSS supplemented media to simulate an

androgen-deprived environment. Confocal microscopy analysis identified that LNCaP control cells had a diffused distribution of AR between cytoplasm and nucleus (Figure 3.3, panel A). As expected, treatment with DHT (1nM for 2hrs) (Figure 3.3, panel A) resulted in AR translocation to the nucleus, without marked effect on microtubule structure. Treatment with the MDV (1μM; 24 hrs) increased cytoplasmic AR with no apparent effect on microtubule structural network (Tran et al., 2009a) (Figure 3.3, panel A). CBZ reduced overall AR immunoreactivity, while it sustained nuclear localization of AR regardless of the presence of synthetic androgens (R1881) or MDV. Treatment of VCaP cells for 24hrs with MDV, followed by pulsing with DHT (2hrs) was visualized by confocal microscopy (Figure 3.4, panel A). AR localization was primarily confined to the nucleus, indicating that MDV was unable to completely block the androgen-mediated AR nuclear translocation (Figure 3.4, panel A). Treatment of VCaP with CBZ for 96hrs leads to AR nuclear localization independently of androgens (Figure 3.3, panel A; Figure 3.4, panel A). There was a significant impact by CBZ treatment on the microtubule structure with clear tubule bundling on the periphery of the cell and complete loss of fibrous microtubule network appearance (Figure 3.3, panel A; green fluorescence). The effects on the microtubule appearance were consistently detected in response to CBZ, associated with remarkable multi-nucleation in both cell lines (Zoom images) (Figure 3.3, panel A; Figure 3.4, panel A). The PC3v567es cells expressing the AR variant v567es (resistant to CBZ) (Figure 3.1, panels A and B) were treated with CBZ and subjected to confocal microscopy. For the PC3567es AR variant cells, CBZ exerts the bundling effect on microtubule structures (Figure 3.5, panel A); the taxane also reduced AR levels but it fails to sustain nuclear localization of AR variant v567es. Extensive multi-nucleation can be observed for PC3v567es cells treated with CBZ alone or in combination with androgens.

To determine the effect of CBZ treatment on AR expression, LNCaP and VCaP prostate cancer cells were treated for 24, 48, or 72hrs with CBZ alone or pulsed with DHT for 3hrs prior to cell lysis and Western blot analysis. CBZ treatment for 72hrs markedly reduced AR protein levels in the androgen sensitive prostate cancer cell lines, LNCaP and VCaP (Figure 3.3, panel B and Figure 3.4, panel B), respectively. Expression of β-tubulin was not affected. CBZ treatment (48hrs) led to caspase-3 cleavage indicative of apoptosis induction in LNCaP cells (Figure 3.3, panel B). To confirm the effect of CBZ on AR localization *in vitro*, we next performed subcellular fractionation analysis in LNCaP cells after exposure to CBZ (24, 48, or 72hrs), alone or pulsed with R1881 (1nM for 2hrs). Androgens predictably increased nuclear AR expression compared to controls (Figure 3.3, panel C). CBZ treatment for 24-48hrs decreased AR levels in LNCaP cells, while there was nuclear retention of AR (Figure 3.3, panel C); by 72hrs AR levels were diminished in both cytosolic and nuclear fractions (Figure 3.3, panel C).

We subsequently analyzed the effect of CBZ on the expression of AR regulated genes in androgen responsive prostate cancer cells as well as in CRPC cells. To establish that the effect of CBZ on AR expression was a consequence of transcriptional inhibition, the AR mRNA levels were evaluated. LNCaP cells were treated with CBZ (24, 48 or 72hrs) alone or pulsed with DHT for 2hrs prior to mRNA extraction. CBZ treatment (24hrs) led to downregulation of AR mRNA (Figure 3.3, panel D). Moreover CBZ significantly inhibited expression of the AR-regulated gene PSA and AR interactor FOXO1, (Figure 3.3, panels E and F), respectively indicating targeting of AR transcriptional activity by CBZ. A similar effect of Cabazitaxel on AR regulated gene expression was also found for the VCaP cells (Figure 3.4, panels C, D and E).

Cabazitaxel Causes Multi-nucleation in Prostate Cancer Cells by Targeting Kinesins

As chemotherapeutic agents, taxanes can effectively target the microtubules and the mitotic spindle apparatus thus blocking cellular division by inducing G2M arrest and apoptosis (Rath & Kozielski, 2012). To determine whether exposure of prostate cancer cells to Cabazitaxel results in the characteristic G2M arrest consequential to microtubule stabilization, we performed confocal microscopy analysis. As shown on Figure 3.6 (panel A), three human prostate cancer cell lines with different AR status, DU145 cells (AR negative), PC3v7 AR variant, LNCaP (mutant AR) and VCaP (full length AR) cells, exhibit increased incidence of multi-nucleation in response to CBZ treatment. In the LNCaP cells, there was a disruption of the mitotic spindle and mono-astral spindle formation (Figure 3.6, panel A.) Overexpression of certain mitosis promoting kinesins can facilitate taxane resistance due to their microtubule depolymerizing action. To establish the targeting of "pro-mitotic" kinesins by CBZ, we next profiled the expression of a subset of mitotic kinesins in human prostate cancer cell lines (Figure 3.6, panel B). MCAK kinesin plays an important role in facilitating spindle pole capture and also acts as a microtubule de-polymerizing factor. HSET functionally mediates cytokinesis. LNCaP cells express relatively low kinesin levels, while VCaP cells exhibit high expression of both proteins (Figure 3.6, panel B). DU145 and PC3v567es have high expression of MCAK but completely lack HSET protein expression (Figure 3.6, panel B). In response to CBZ (24, 48 or 72hrs) alone or under androgenic pulse, there was a transient increase in expression of MCAK and HSET kinesins within the first 24hrs in the VCaP cells; by 72hrs of treatment kinesin levels were significantly down regulated (Figure 3.6, panel C and Figure 3.7, panel A). In addition there was a significant decrease in MCAK (KIF2C) mRNA expression in LNCaP and VCaP cells (Figure 3.6, panel D and Figure

3.7, panel C), and in HSET (KIFC1) mRNA levels for VCaP cells (Figure 3.7, panel B) in response to CBZ. To determine the potential link between action of CBZ on mitotic spindle formation and resistance, we subsequently examined pericentrin (marker of centrosomes) expression in the Cabazitaxel-resistant prostate cancer PC3v567es and VCaP cells and the CBZ sensitive CRPC 22Rv1 cells. As shown on Figure 3.6 (panel E), PC3v567es cells in response to CBZ (48 and 72hrs) exhibited centrosome clustering and amplification accompanied with severe multi-nucleation, independent of androgenic presence. The VCaP cells (tetraploid) also exhibited prominent multi-nucleation and centrosome clustering and amplification (detected by pericentrin) in response to CBZ; these effects on the mitotic dynamics were not influenced by the status of androgen axis (presence of androgens or antiandrogen, MDV) (Figure 3.6, panel F). In the CRPC 22Rv1 cells, CBZ treatment led to multi-nucleation but not centrosomal amplification (Figure 3.8, panel A).

In Vivo Novel Action of Cabazitaxel in Models of Advanced Prostate Cancer via Induction of Mesenchymal-Epithelial Transition (MET) and Glandular Differentiation

To define the physiological significance of our *in vitro* findings we investigated the cellular phenomena driving the anti-tumor action of Cabazitaxel in an *in vivo* model of advanced prostate cancer. Previous work in our laboratory established a double transgenic mouse model of TRAMP crossed with mice expressing a conditional dominant negative TGFβRII (Pu et al., 2009). This TRAMP/DNTGFβRII model is characterized by aggressive tumor progression to metastasis driven by EMT. The consequences of Cabazitaxel treatment on the phenotypic landscape and growth dynamics of advanced prostate cancer were evaluated in prostate tumors from (N=10) 16-18-wks male mice (castrate versus non-castrate groups) receiving treatment for 14 days with pharmacological dose of CBZ (described in Figure 3.9; panels A and B). CBZ

treatment led to a significant decrease in the body weight but not prostate weight ($P<0.005$) (Figure 3.9, panels C and D respectively; $P<0.005$). At 18-20wks, TRAMP+DNTGFBRII+ mice progressed to aggressive poorly differentiated prostate cancer (Pu et al., 2009); Histopathological evaluation (H&E staining) revealed that treatment with CBZ alone or in combination with androgen depletion restored the glandular structures and luminal secretions of the prostate epithelium compared to controls (VHC) (Figure 3.10, panel A).

To determine the impact of CBZ treatment on the tumor growth kinetics, apoptosis and cell proliferation were evaluated in the transgenic mouse model of androgen-sensitive prostate cancer, in the presence of an intact or depleted androgen axis. Evaluation of prostate tumor cell proliferative capacity based on Ki-67 and phospho-Histone 3 (pH3) immunoreactivity revealed an increased proliferative activity in response to CBZ alone, while castration-induced-androgen deprivation resulted in a significant decrease in the proliferative index (Figure 3.10, panels A and D). To correlate the effect by CBZ on the mitotic spindle with resulting multi-nucleation (observed in vitro; Figure 3.6), expression of the nuclear protein phospho-H3 histone, was examined in prostate tumors. As shown on Figure 3.10 (panel B), CBZ resulted in distinct multi-nucleation among prostate tumor glands from CBZ-treated mice, compared to VHC controls (Figure 3.10, panel B, arrows). The incidence of apoptosis as reflected by an increased number of TUNEL positive cells in prostate tumors was induced after castration-induced androgen withdrawal; at 48hrs-post castration-induced apoptosis reaches a peak compared to controls ($P<0.005$) (Figure 3.10, panels A and C). Treatment of either intact or castrate mice (androgen-depleted), with CBZ (2wks) failed to induce significant apoptosis compared to VHC control mice (Figure 3.10, panels A and C). The incidence of proliferation as reflected by an increased number of Ki67

positive cells in prostate tumors was decreased after castration-induced androgen withdrawal alone or in combination with CBZ treatment ($P<0.005$) (Figure 3.10, panels A and D).

The potential contribution of EMT-MET cycling to the glandular formation and phenotypic reversion to differentiated prostate epithelium in response to the taxane treatment was interrogated by profiling the expression of EMT markers, E-cadherin and N-cadherin in serial sections of prostate tumors from treated and untreated mice. Figure 3.11, panel A indicates representative images of immunoreactivity analysis of the EMT landscape. Certain populations of tumor epithelial cells exhibited strong E-cadherin immunoreactivity paralleled by decreased N-cadherin expression in response to CBZ, supporting an effect by the drug on reversing EMT (Figure 3.11, panel A). Our previous work established that prostate tumors from TRAMP+DNTGFβRII mice exhibit marked acceleration of progression to metastatic lethal disease via changes in the tumor microenvironment driven by increased inflammation and enhanced EMT (Pu et al., 2009). The present study demonstrates that Cabazitaxel alone or in combination with androgen-depletion reversed EMT to MET as reflected by elevated E-Cadherin and decreased N-Cadherin immunoreactivity (Figure 3.11, panel A). Intense nuclear immunoreactivity for AR was detected in the prostate tumors from control (VHC) male mice; upon castration-induced androgen ablation there was a marked reduction in AR nuclear presence associated with a diffused localization to the cytoplasm (Figure 3.11, panels B and C). Treatment of intact mice with CBZ resulted in a significant nuclear AR localization, compared to androgen-depletion mediated cytoplasmic translocation (Figure 3.11, panels B and C). Prostate tumor epithelial cells in castrate mice treated with CBZ (for 2wks) exhibited a reduced AR expression with a significant inhibition of AR nuclear

localization compared to VHC controls or CBZ-alone treated mice (Figure 3.11, panels B and C).

The kinesin immunoreactivity profile in prostate tumors from the transgenic mouse model revealed decreased MCAK expression in response to CBZ (given as a single agent), compared to VHC controls, castration-androgen depletion or combination of CBZ with castration (Figure 3.11, panel B). Impairing the androgen axis (castration) alone or in combination with CBZ led to increased kinesin expression (Figure 3.11, panel B). We subsequently examined the effect of CBZ on the gene expression profile of critical EMT regulators in the two androgen-sensitive human prostate cancer cell lines, VCaP and LNCaP RT-PCR analysis of mRNA expression for E-cadherin, Vimentin and Twist1 (Figure 3.11, panels D, E and F) in VCaP cells after CBZ treatment, demonstrated a significant downregulation in all three genes within 24hrs of treatment, that was not affected by the presence of DHT. A similar profile of decrease in mRNA expression for EMT effectors in response to CBZ was observed in LNCaP cells (Figure 3.11, panels G, H and I).

The *in vivo* anti-tumor effect of CBZ alone or in combination with anti-androgen (MDV) against the human CRPC 22Rv1 xenografts is shown on Figure 3.13 (Dosing regimen described in Figure 3.12). CBZ alone decreased tumor mass in tumor-bearing mice (when compared initiation vs termination of treatment per individual mouse), although this failed to reach statistical significance (Figure 3.13, panels A and B). CBZ alone or in combination with MDV3100 led to a significant reduction in body weight (Figure 3.12, panel C). Tumor specimens from control (VHC) and treated mice were subjected to immunohistochemical analysis for the tumor growth kinetics, apoptosis and cell proliferation, vascularity, AR expression and localization, and kinesin expression. Our data indicate that treatment with CBZ alone or in combination with MDV results in

significant induction of apoptosis (Figure 3.13, panels C and D). The antiandrogen given as a single agent failed to induce apoptosis in the CRPC 22Rv1 tumors. We also observed a significant reduction in tumor vascularity in response to CBZ alone that was reversed by the combination treatment with the anti-androgen (Figure 3.13, panels C and E). There was a significant increase in Ki-67 immunoreactivity in response to CBZ treatment alone, while CBZ in combination with MDV3100 suppressed prostate tumor proliferation (Figure 3.13, panels C and F). Treatment of CRPC 22Rv1 cells with CBZ downregulates MCAK protein levels in a temporal correlation with loss of E-cadherin expression driven by CBZ, in the presence or absence of androgens (R1881) (Figure 3.13, panel G). We observed a pattern of transient changes in AR expression levels in CRPC 22Rv1 cells in response to CBZ; As shown on Figure 3.13 (panel G) there was temporal reduction in full length AR levels within 24 hrs of treatment followed by a significant increase at 48hrs post-treatment, compared to untreated control cells (Figure 3.13, panel G). No marked changes were detected for the AR variant V7 in response to CBZ.

The impact of Cabazitaxel on EMT landscape in the CRPC xenografts was profiled with three marker proteins, E-cadherin, N-cadherin and ZEB-1. As shown on Figure 3.14 (panel A), CBZ treatment resulted in increased E-cadherin, while it decreased N-cadherin and ZEB1 immunoreactivity indicating abrogation of EMT programming in response to the taxane. These *in vivo* phenotypic findings in the CRPC xenograft model are consistent with the effect of the drug in the transgenic model of EMT-driven prostate tumor progression (Figure 3.11). Also shown on Figure 3.14 is the effect of CBZ on reducing kinesin immunoreactivity, for both MCAK and HSET in serial sections of CRPC xenograft tumors (Figure 3.14, panel B). MDV3100 treatment had no significant effect on HSET kinesin levels, while it reduced MCAK expression. CBZ

treatment reduced AR expression but it maintained a strong AR nuclear localization in the CRPC 22Rv1 tumors compared to VHC control (Figure 3.15, panel A). In contrast, the antiandrogen, MDV3100 induced a marked cytoplasmic distribution of AR (Figure 3.15, panel A). Quantitative analysis of the cellular distribution of AR in response to treatment revealed a significant increase in high intensity nuclear AR in the CBZ-treated CRPC tumors compared to MDV3100 or combination of CBZ and the antiandrogen (Figure 3.15, panel B).

Conclusions

Microtubule-stabilizing chemotherapeutic agents such as Docetaxel and Paclitaxel have been shown to inhibit AR nuclear localization and activity in human prostate cancer, an action that correlates with the therapeutic response in patients as discussed in Chapters 1 and 2 (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a). Our lab and others have reported the effect of Docetaxel on preventing nuclear translocation of the AR from the cytoplasm in prostate cancer, (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a). In sharp contrast, the present results demonstrate that in *in vitro* and *in vivo* models of androgen sensitive and CRPC advanced prostate tumors Cabazitaxel chemotherapy maintains AR nuclear localization, while it inhibits AR expression. Moreover, my data demonstrate that the sensitivity of human prostate cancer cells to Cabazitaxel was not associated with AR variant status. Thus PC3v567es (androgen independent with AR variant) and VCaP (androgen responsive with AR full length) cells exhibited comparable resistance to Cabazitaxel treatment. These findings are in accord with recent evidence indicating that the therapeutic antitumor effect of Cabazitaxel

proceeded via an AR-independent mechanism (van Soest et al., 2014). The mechanisms driving therapeutic cross-resistance to taxanes and antiandrogens in CRPC involve microtubule stabilization and inhibition of AR activity and nuclear localization by interfering with tubulin-AR association (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; Mistry & Oh, 2013; M.-L. Zhu et al., 2010a). Recent studies identified new signaling effectors conferring mechanistic resistance to taxane chemotherapy in CRPC, including overexpression of ERG (Galletti et al., 2014) and activation of the GATA2-IGF2 signaling axis (Vidal et al., 2015).

Centrosome amplification promotes transient spindle multipolarity during mitosis and correlates with tumor aggressiveness and chemotherapeutic resistance (Ogden et al., 2014; Ogden, Rida, & Aneja, 2012, 2013). The phenomenon of multi-nucleation that predominated the Cabazitaxel- treated prostate cancer cells regardless of their AR variant status, as well as the ability of the drug to induce centrosome amplification in the resistant cancer cell lines, provides a shift in our understanding of therapeutic resistance to taxane chemotherapy. Indeed, my work identified the mitotic centromere-associated kinesin (MCAK) is a direct target of Cabazitaxel in both androgen-sensitive and CRPC tumors. The kinesin spindle protein (KSP) is a molecular motor that crawls along the microtubules to assist cell division (also known Eg5) and different mitotic kinesins serve specific functions during cell division. Although a specific KSP inhibitor has been evaluated in Phase II clinical trials for relapsed multiple myeloma, the clinical data indicate that such KSP inhibitors (Ispinesip) might be used in combination with proteasomal inhibitors or immuno-modulators as frontline therapy for myeloma patients. In prostate cancer, early-phase clinical trials in CRPC patients using the first generation Eg5 inhibitor, Ispinesip, have met limited success (Blagden et al, 2008; Beer et al; 2008). The kinesin spindle protein KIF11 (Eg5) has been shown to be functionally involved in

prostate cancer cell growth *in vitro* and *in vivo* models (Hayashi et al, 2008; Davis et al; 2006). In accordance with my present findings, MCAK was identified as a potential mitosis phase target in prostate cancer that was overexpressed in CRPC gene expression datasets (Sircar et al., 2012).

My results demonstrate that Cabazitaxel can effectively stabilize microtubule structures in androgen-sensitive and castration-resistant prostate cancer cells in accordance with the current evidence on the action of this taxane chemotherapeutic agent (Azarenko et al., 2014; Vrignaud et al., 2013). With the knowledge that Cabazitaxel was designed to bind with β-tubulin subunits and stabilize their interaction preventing depolymerization of the structure, once assembled, the microtubules cannot disassemble leading to mitotic blockade. In contrast to Docetaxel that induced microtubule stabilization leading to classic G2M arrest and apoptosis as well as AR cytoplasmic localization, I report that Cabazitaxel treatment induces severe multi-nucleation and centrosome clustering that led to the development of mono-astral spindle formations in the androgen-sensitive prostate cancer cells. These aberrations of mitosis lent insight that in addition to microtubule stabilization, Cabazitaxel treatment targets expression of the mitotic kinesins which facilitate this process (Nakouzi et al., 2014). The inhibitory effect of Cabazitaxel on protein and mRNA expression of MCAK and HSET kinesins provides an initial mechanistic insight into the ability of a microtubule targeting chemotherapy to effectively target a mitotic kinesin and consequently interfering with AR transport across the microtubules, leading to tumor suppression (illustrated on Figure 3.15, panel C). The concept that kinesins determine the therapeutic targeting and response to Cabazitaxel in advanced prostate cancer gains strong support from clinical evidence documenting a correlation between

overexpression of MCAK with tumor progression to advanced disease in CRPC patients (Sircar et al., 2012);

Utilizing a transgenic mouse model of androgen-responsive prostate cancer that is driven by EMT to advanced disease, we analyzed the consequences of Cabazitaxel on EMT in prostate tumor progression *in vivo*. At 18-20wks of age, the transgenic mouse model is characterized by aggressive and poorly differentiated prostate tumors (Pu et al 2009). I made the intriguing observation that prostate tumors from mice treated with Cabazitaxel alone or in combination with androgen deprivation, exhibited a phenotypically re-differentiated prostate epithelium with intact luminal secretions. One could argue that the plasticity afforded to a fully differentiated epithelium by Cabazitaxel treatment, allows individual cells to de-differentiate into mesenchymal-like derivatives in reversible phenotypic transformative process. Thus, during several rounds of EMT and the reverse process, MET allow for the formation of differentiated glandular epithelial structures in response to Cabazitaxel. This gains indirect support by the observation that the incidence of apoptosis in prostate tumors in response to Cabazitaxel, or the combination treatment of Cabazitaxel and androgen-depletion was lower compared to significant apoptosis induced by androgen deprivation. In an interesting twist of growth kinetics, Cabazitaxel increased the proliferative activity of prostate tumor cell populations similar to the intact control mice. Considering that a dynamic EMT-MET cycling has been functionally implicated in the formation of complex epithelial tissues (Moreno-Bueno et al., 2008), my data strongly support the ability of Cabazitaxel to induce epithelial glandular differentiation of aggressive advanced prostate tumors via reversal of EMT to MET, possibly driving phenotypic response to taxane chemotherapy.

This work provides a molecular basis for the emerging role of AR variants in predicting therapeutic resistance of advanced CRPC to MDV (Antonarakis et al., 2014)

and taxanes in experimental models of CRPC (Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, Plymate, et al., 2014). Overexpression of AR splice variants may preclude patients from undergoing Cabazitaxel and antiandrogen (MDV) combination therapy due to therapeutic resistance potentially driven by centrosome clustering. An insight into this cross-resistance is provided by our observations that combination of Cabazitaxel and MDV (at high doses) leads to a significant growth stimulatory response in the CRPC 22Rv1 cells (harboring AR variants), while in the androgen-sensitive VCaP cells (full length AR), MDV treatment was able to overcome Cabazitaxel resistance. A molecular explanation for this effect is provided by a recent study reporting that MDV treatment of CRPC 22Rv1 cells has no effect on AR V7 variant expression, while it upregulated V7 in the VCaP cells (Schweizer et al., 2015).

In summary, the present study provides the first evidence that Cabazitaxel chemotherapy impairs prostate tumor progression by reversing EMT to MET towards phenotypically differentiated prostate glandular/luminal architecture, via disrupting the kinesin network. Cellular re-differentiation via MET conversions may account for the similarities in the phenotypic landscape between primary tumors and bone distant metastatic lesions that can dictate their therapeutic resistance to taxane-based chemotherapy and antiandrogens. My findings indicate that resistance to Cabazitaxel can be overcome by concurrently targeting the androgen/AR axis in the androgen-responsive prostate tumors, while in CRPC antiandrogens may further enhance centrosome amplification-driven resistance to Cabazitaxel, provide a new molecular basis for understanding therapeutic cross-resistance in prostate cancer patients failing treatment with Cabazitaxel chemotherapy and antiandrogens.

Figure 3.1: Human Prostate Cancer Cell Response to Cabazitaxel and AR Targeting. Panel A, Dose response analysis of human prostate cancer cells, DU-145, PC3, PC3v567es, 22Rv1, LNCaP, LNCaPTβRII and VCaP to increasing concentrations of Cabazitaxel (10-500nM) for 96hrs. Cell viability was evaluated by the MTT assay. Panel B, Time course of loss of cell viability in response to Cabazitaxel (100nM, 24-120hrs).

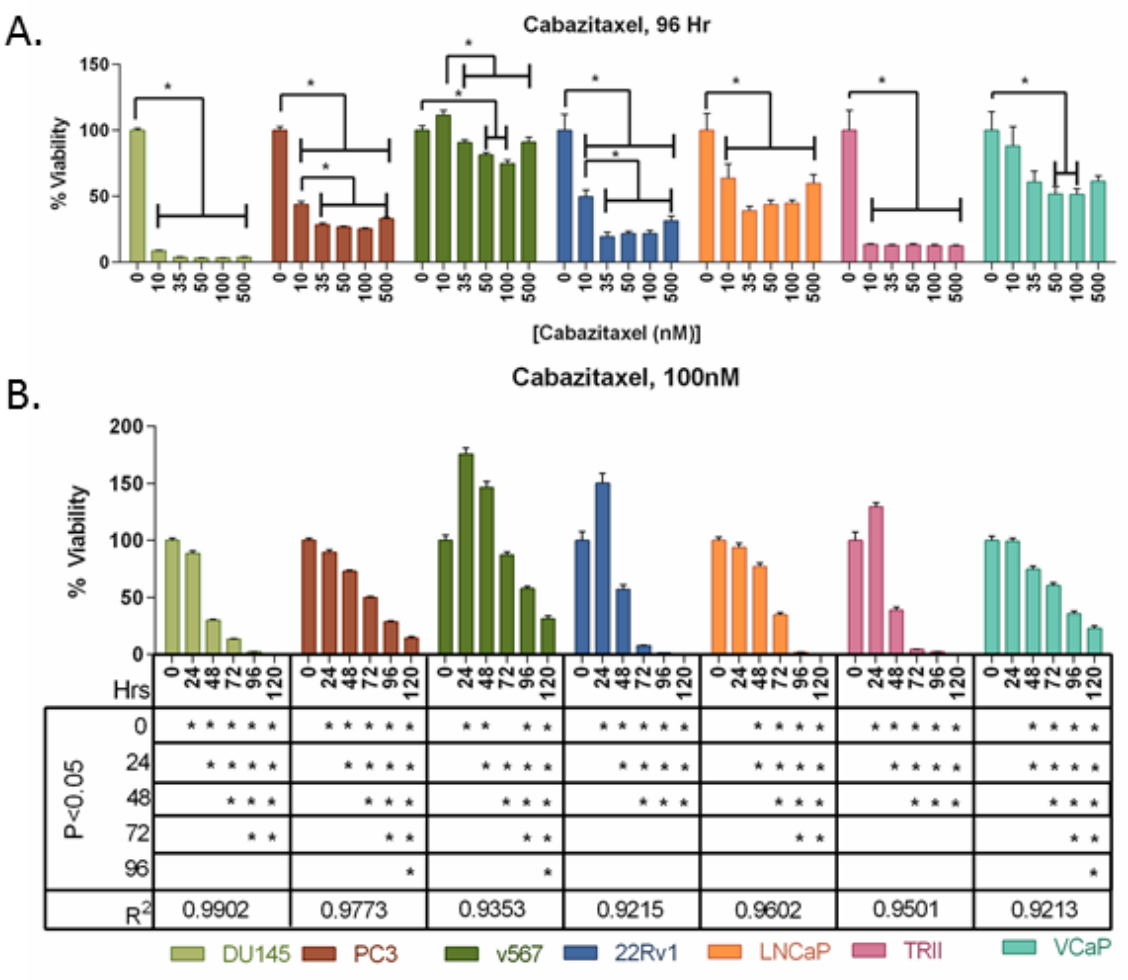


Figure 3.1 continued, Human Prostate Cancer Cell Response to Cabazitaxel and AR Targeting. Panel C, Effect of Cabazitaxel (100nM) treatment alone or in combination with antiandrogen MDV3100 (MDV, 1-10µM) on prostate cancer cell viability. Panels D and E, Results of cell cycle analysis of PC3v567es cells indicating that CBZ alone induces G2 and S phase arrest. Panels F and G, Cell cycle analysis of CRPC 22Rv1 cells after treatment with CBZ, synthetic androgen (R1881) and MDV as single agents or in combination. CBZ induces G2 and S phase arrest alone or in combination with MDV. Data represent the mean of three independent experiments analyzed in duplicate ± SEM; * indicates P<0.05 as determined by one way ANOVA.

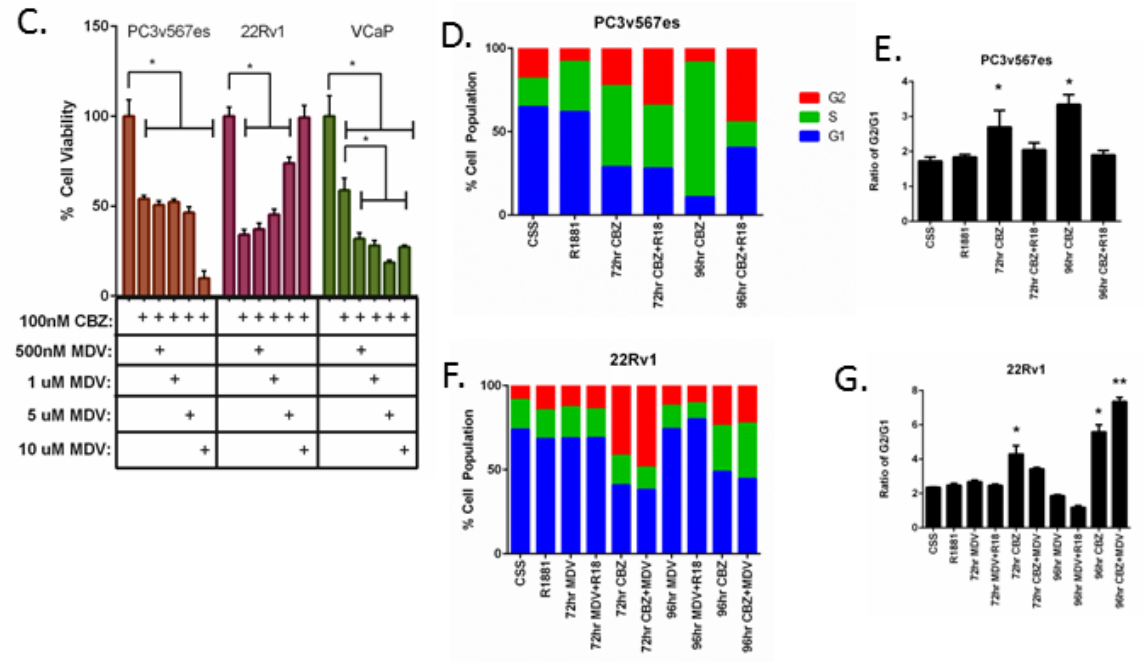


Figure 3.2. Dose Response Analysis of Human Prostate Cancer Cells to Antiandrogen Enzalutamide (MDV3100) and Docetaxel. Prostate cancer cell lines were treated for 24hrs with increasing concentrations of MDV/DTX/Combination and cell viability was assessed using MTT assay. Data represent the mean of three independent experiments analyzed in duplicate ± SEM; * indicates P<0.05 as determined by one way ANOVA. Panel A, reveals the dose response to MDV treatment (0.1nM, 10nM, 1µM, 10µM). Panel B, reveals the dose response to Docetaxel (DTX) treatment (0.1, 0.5, 1, 2.5, 5 µM).

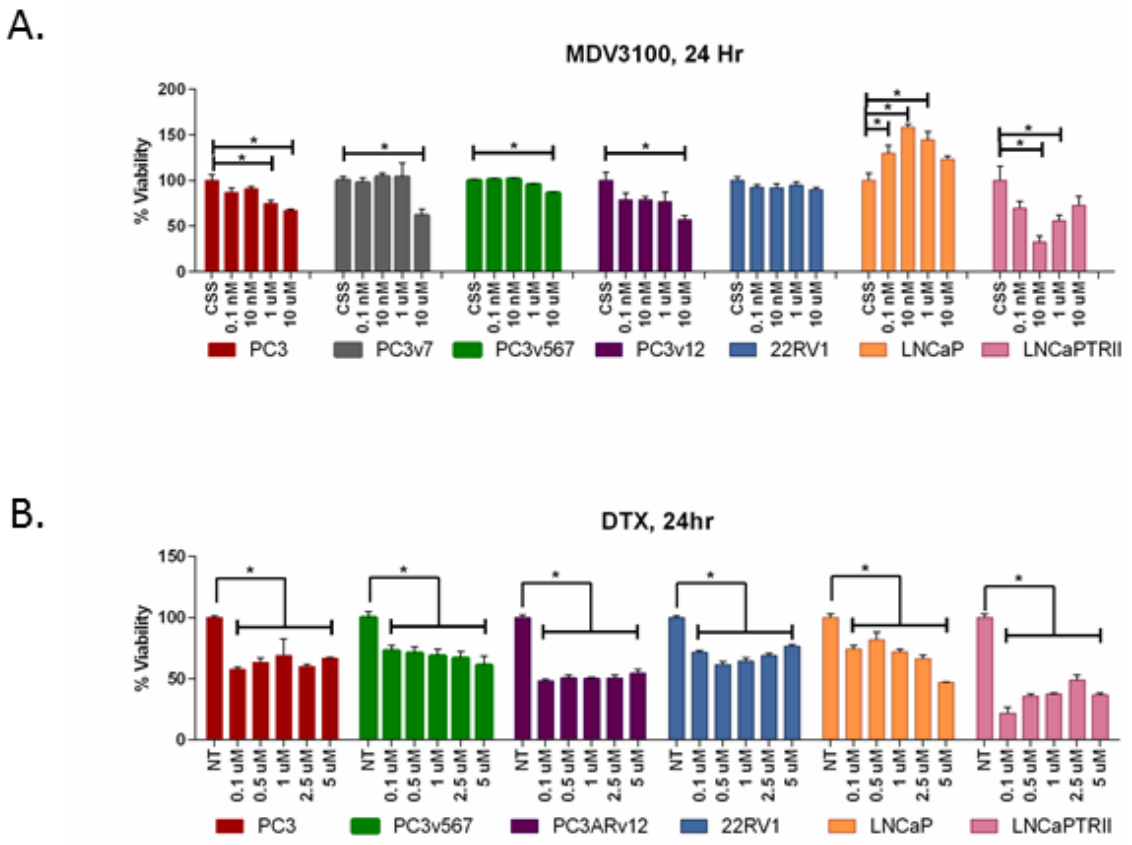
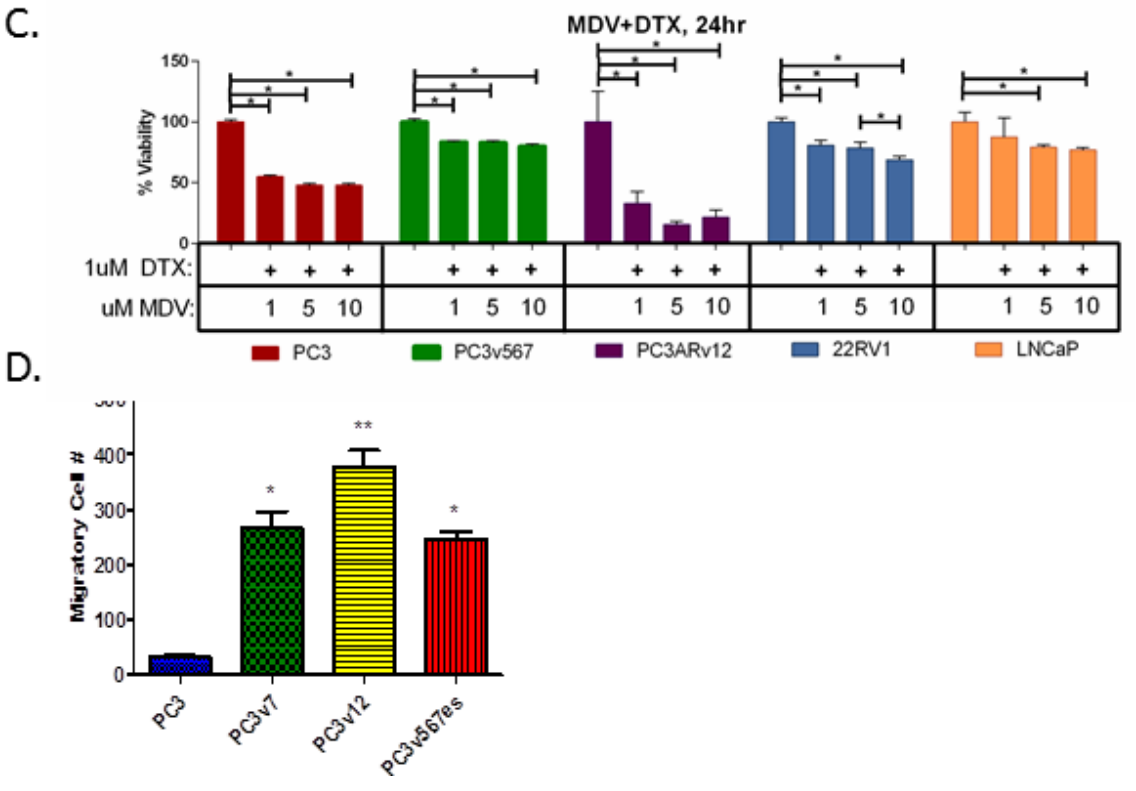


Figure 3.2 continued, Dose Response Analysis of Human Prostate Cancer Cells to Antiandrogen Enzalutamide (MDV3100) and Docetaxel. Panel C reveals the dose response of combination of MDV (1, 5, 10 µM) with DTX (1µM). Panel D reveals the consequences of forced overexpression of AR variants on PC3 prostate cancer cell migration using wound healing assay. The migration potential of PC3 parental, PCv3567, PC3v7 and PCv12 cells was analyzed. Data represent the mean of quantitated distribution of AR (N-20) in response to various treatments ± SEM; * indicates P<0.05 as determined by one way ANOVA.



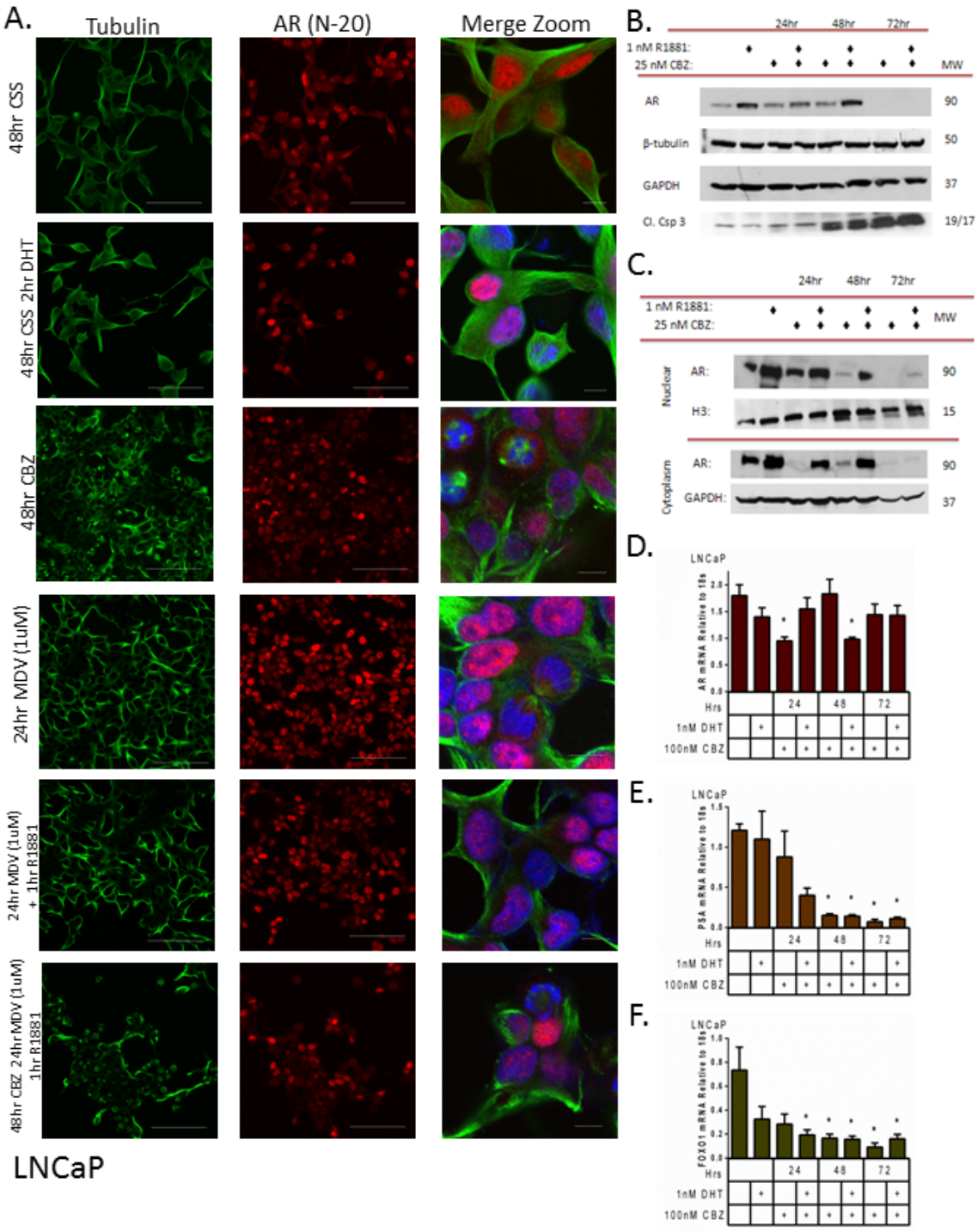

A.
Tubulin
AR (N-20)
Merge Zoom
48hr CSS
48hr CSS 2hr DHT
48hr CBZ
24hr MDV (1uM)
24hr MDV (1uM) + 1hr R1881
48hr CBZ 24hr MDV (1uM) 1hr R1881
LNCaP
B.
24hr
48hr
72hr
1 nM R1881:
25 nM CBZ:
MW
AR
β-tubulin
GAPDH
Cl. Csp 3
90
50
37
19/17
C.
Nuclear
Cytoplasm
AR:
H3:
GAPDH:
15
D.
LNCaP
AR mRNA Relative to 18s
Hrs
1nM DHT
100nM CBZ
E.
LNCaP
PSA mRNA Relative to 18s
F.
LNCaP
FOXO1 mRNA Relative to 18s

Figure 3.3: Effect of Cabazitaxel on AR Localization and Gene Expression in Prostate Cancer Cells. Panel A, Representative confocal images of AR expression in androgen sensitive human prostate cancer cells, LNCaP. Cells were treated with either DHT (1nM for 3hrs), MDV3100 (1µM for 24 hrs), and CBZ (25nM for 96hrs) as single treatments, or in combination and subjected to fluorescent labeling for tubulin, AR (N-20) and DAPI (nucleus). Images were visualized under 40x oil immersion confocal microscopy, scale bars for Tubulin and AR (N-20) are 100 µM and scale bars for Zoom Merge (10 µM) . Panel B reveals, Expression profile of AR(N-20), β-tubulin, and cleaved caspase-3 in LNCaP cells treated with CBZ (25nM) in the presence or absence of R1881 (1nM) for 24-72hrs. GAPDH expression was used as a loading protein control. There was a significant decrease in AR within 24hrs of CBZ treatment, paralleled by increased caspase-3 cleavage. Panel C, subcellular fractionation of LNCaP prostate cancer cells, after treatment with CBZ (25nM), in the presence or absence of R1881 (1nM). Western blots were probed for AR (N-20), Histone H3 and GAPDH. Panels D, E and F, Effect of CBZ (25nM) on AR mRNA expression, its target gene PSA, and FOXO1 in LNCaP prostate cancer cells (in presence / absence of androgens). RT-PCR analysis shows a significant decrease in mRNA expression for the three genes, AR, PSA, and FOXO1 by CBZ treatment. Data represent the mean of three independent experiments analyzed in duplicate ± SEM; * indicates $P<0.05$ as determined by multiple comparisons of two way ANOVA.

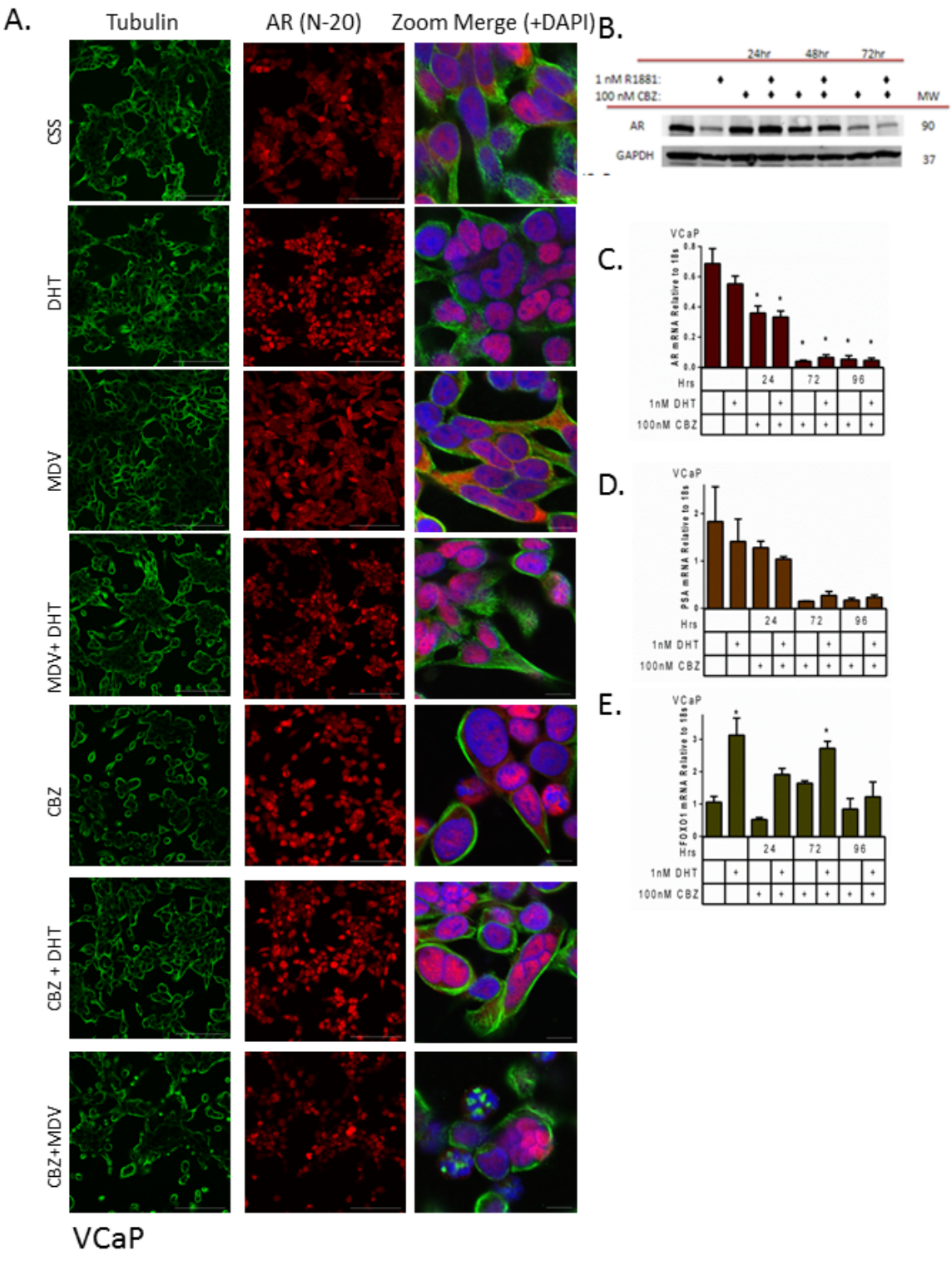

A.
Tubulin
AR (N-20)
Zoom Merge (+DAPI)
CSS
DHT
MDV
MDV+ DHT
CBZ
CBZ + DHT
CBZ+MDV
VCaP
B.
24hr
48hr
72hr
1 nM R1881:
100 nM CBZ:
MW
AR
GAPDH
90
37
C.
VCaP
AR mRNA Relative to 18s
Hrs
24
72
96
1nM DHT
100nM CBZ
D.
VCaP
PSA mRNA Relative to 18s
Hrs
24
72
96
1nM DHT
100nM CBZ
E.
VCaP
FOXO1 mRNA Relative to 18s
Hrs
24
72
96
1nM DHT
100nM CBZ

Figure 3.4. Combined Effect of Cabazitaxel and Enzalutamide on AR Expression and Localization in VCaP Cells. Panel A, Representative confocal images of AR expression and localization in VCaP cells, control (CSS), and in response to MDV (1 μM), in the presence of DHT (1nM for 3hrs), or CBZ (100nM for 96hrs) as single treatments or in combination; Cells were subjected to immunofluorescence staining for tubulin, AR (N-20) and DAPI (nuclear presence). Images were captured under 40x (oil-immersion) objective; scale bars are 100μM for Tubulin and AR (N-20) and for Zoom Merge (+DAPI) scale bars are 10μM. Panel B reveals Western blot analysis of AR (N-20), VCaP prostate cancer cells treated with CBZ (100nM) with or without androgens (R1881, 1nM). GAPDH expression was used as loading control. Panels C, D and E represent RT-PCR analysis of mRNA expression for AR, PSA and FOXO1 genes in response to CBZ (100nM), in the presence or absence of R1881. Data represent the mean of three independent experiments analyzed in duplicate ± SEM; * indicates $P<0.05$ as determined by multiple comparisons of two way ANOVA.

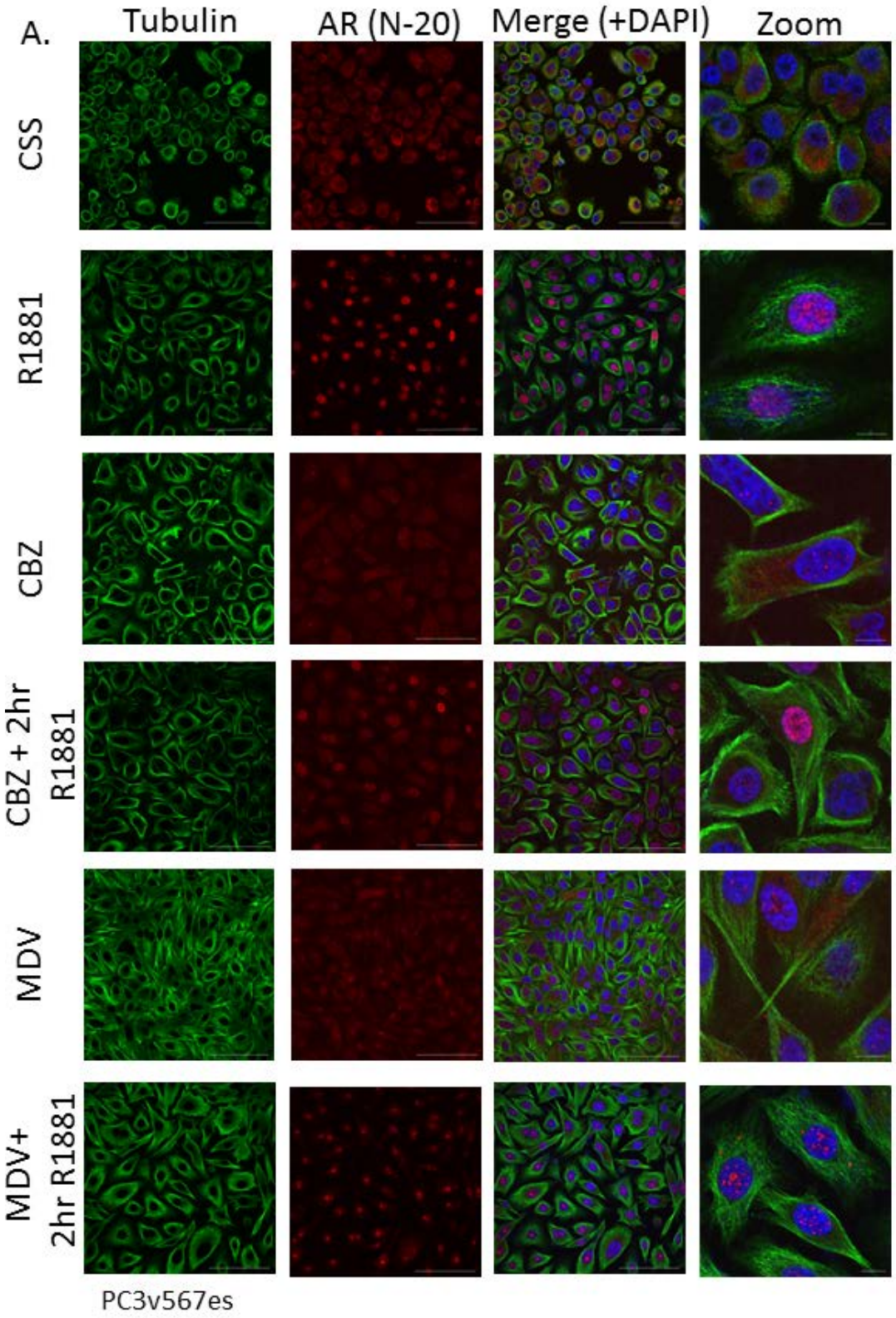

A.
Tubulin
AR (N-20)
Merge (+DAPI)
Zoom
CSS
R1881
CBZ
CBZ + 2hr R1881
MDV
MDV+ 2hr R1881
PC3v567es

Figure 3.5: AR variant Localization and Tubulin Expression in CBZ Resistant PC3v567es Cells. Confocal microscopy was conducted in PC3v567es cells treated with either R1881 (1nM for 3hrs), MDV (1µM, 24hrs), and CBZ (100nM, 96hrs) as single treatments or in combination (CBZ and MDV). Cells were subjected to immunofluorescence staining for tubulin, AR (N-20) and DAPI (nucleus). Images were captured under 40x oil immersion objective; for Tubulin, AR (N-20) and Merge scale bars are 100 µM. For Zoom Merge, scale bars are 10 µM. CBZ leads to diffused cytosolic distribution of AR variant v567es.

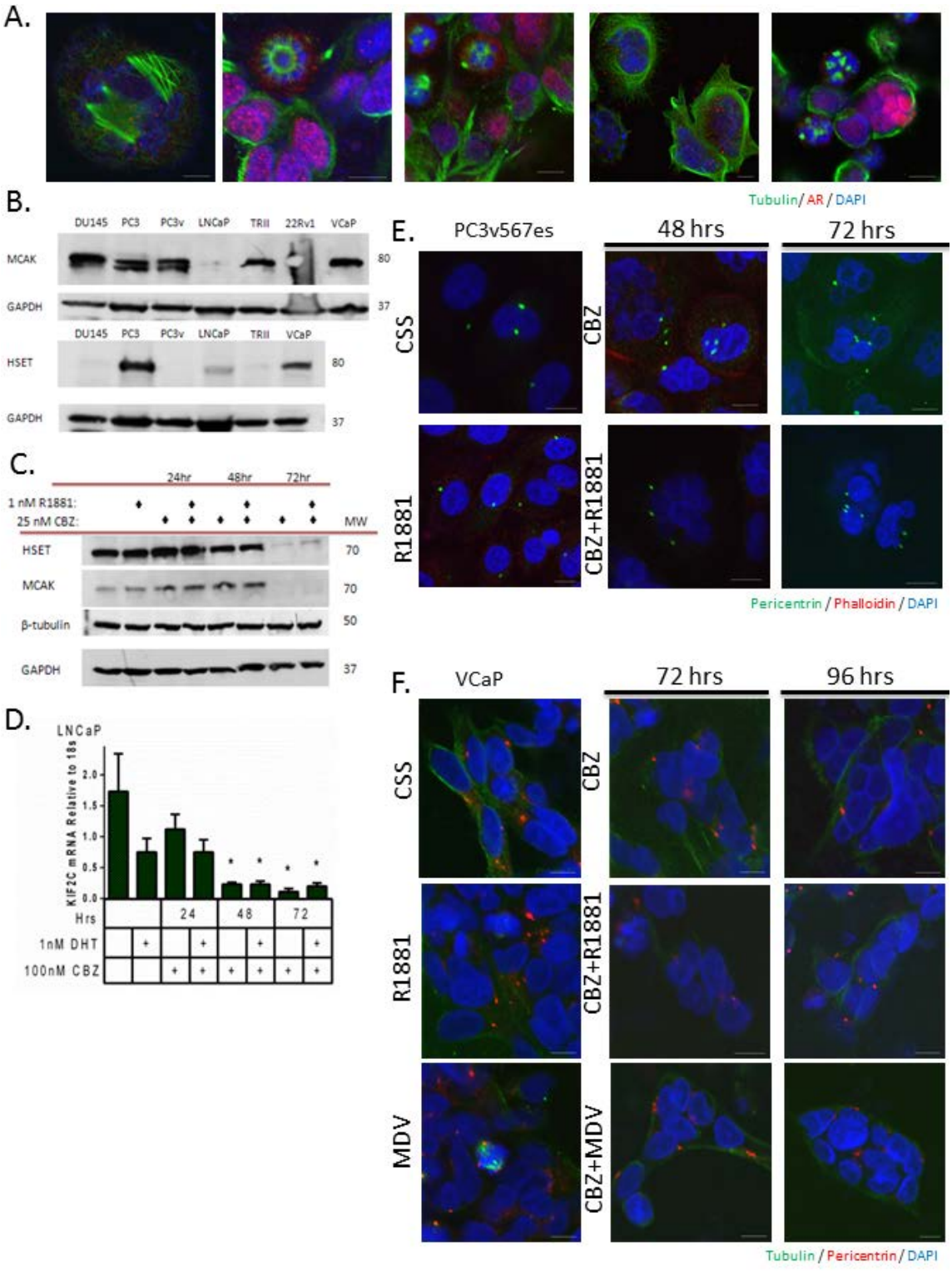

A.
Tubulin/AR/DAPI
B.
DU145 PC3 PC3v LNCaP TRII 22Rv1 VCaP
MCAK
GAPDH
80
37
DU145 PC3 PC3v LNCaP TRII VCaP
HSET
GAPDH
80
37
C.
24hr 48hr 72hr
1 nM R1881:
25 nM CBZ:
MW
HSET
MCAK
β-tubulin
GAPDH
70
70
50
37
D.
LNCaP
KIF2C mRNA Relative to 18s
2.0
1.5
1.0
0.5
0.0
Hrs
24
48
72
1nM DHT
100nM CBZ
E.
PC3v567es
48 hrs
72 hrs
CSS
CBZ
R1881
CBZ+R1881
Pericentrin / Phalloidin / DAPI
F.
VCaP
72 hrs
96 hrs
CSS
CBZ
R1881
CBZ+R1881
MDV
CBZ+MDV
Tubulin / Pericentrin / DAPI

Figure 3.6: Cabazitaxel Results in Multi-nucleation and Centrosome Clustering in Prostate Cancer Cells by Targeting Kinesins. Panel A, From left to right: Human CRPC prostate cancer cells, DU145 treated with CBZ (35nM) for 96hrs exhibit multipolar spindle and microtubule bundling; LNCaP prostate cancer cells treated with CBZ (35nM; 48hrs) exhibit mono-astral spindle formation; LNCaP, prostate cancer cells exhibit multipolar spindle, after treatment with CBZ (72hrs); PC3 cells transfected with AR variant V7 and treated with CBZ exhibit extensive multi-nucleation; VCaP treated with 100 µM CBZ for 96hrs exhibit multipolar spindle and multi-nucleation. Scale bars are 10 µM. Panel B reveals the expression profile of kinesins, KIF2C (MCAK) and KIFC1 (HSET) protein in human prostate cancer cell lines. Panel C, Western blot analysis of HSET, MCAK, and β-tubulin in LNCaP cells treated with CBZ (25nM) alone, or in the presence of androgens (1nM R1881) for 24-72hrs. GAPDH used as loading control. Panel D, RT-PCR analysis of mRNA expression of KIF2C in LNCaP cells in response CBZ for 24, 48 and 72hrs. Panel E, Detection of pericentrin (green), actin (phalloidin-red) and DAPI (blue) by confocal microscopy in PC3v56es cells in response to CBZ for 48 and 72hrs in the presence or absence of androgens. Visualized under 40x oil immersion objective, scale bars are 10 µM. Panel F, Confocal images of VCaP cells treated with CBZ revealing multi-nucleation and centrosome amplification in response to treatment; scale bars are 10 µM. Panel E, Detection of pericentrin (red), tubulin (green) and DAPI (blue) by confocal microscopy in VCaP cells in response to CBZ for 72 and 96hrs in the presence or absence of androgens (1nM) or MDV (1uM) induced multi-nucleation and centrosome amplification in response to treatment. Visualized under 40x oil immersion objective, scale bars are 10 µM.

Figure 3.7. Effect of Cabazitaxel on Kinesin Expression in VCaP Cells. Panel A, Western blot analysis of HSET, MCAK, and GAPDH in VCaP cells treated with CBZ (100nM) alone or in the presence of DHT for 24, 48 and 72 hrs. Panels B and C, RT-PCR analysis of mRNA expression for KIFC1 and KIFC2 (MCAK) genes respectively in VCaP cells. Data represent the mean of three independent experiments analyzed in duplicate ± SEM; * indicates P<0.05 as determined by multiple comparisons of two way ANOVA.

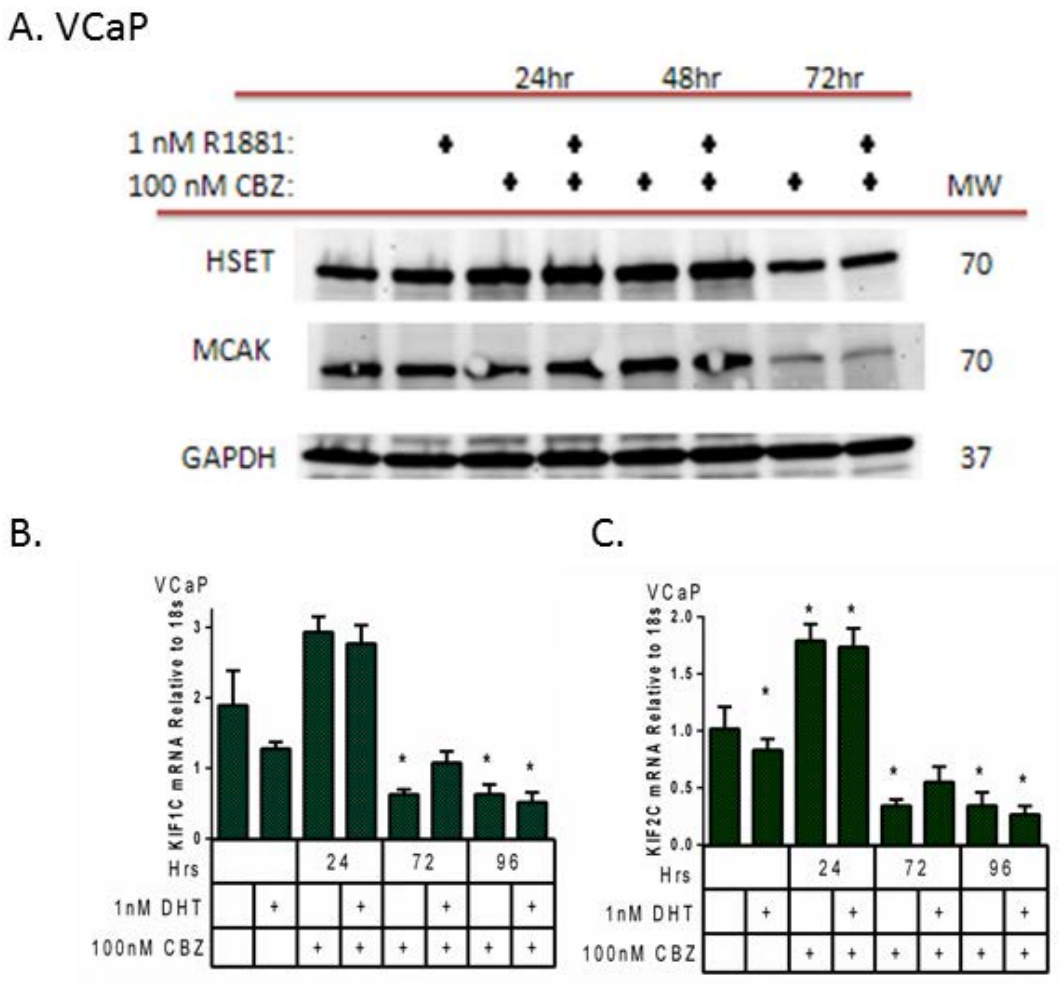


Figure 3.8: Effect of Cabazitaxel on Centrosomal Amplification in CRPC 22Rv1 cells. Detection of pericentrin, tubulin and DAPI by confocal microscopy with immunofluorescence in 22Rv1 cells in response to CBZ (72 and 96hrs) in the presence or absence of androgens (R1881) or anti-androgens (MDV). Images captured under 40x oil immersion objective; scale bars are 10 µM.

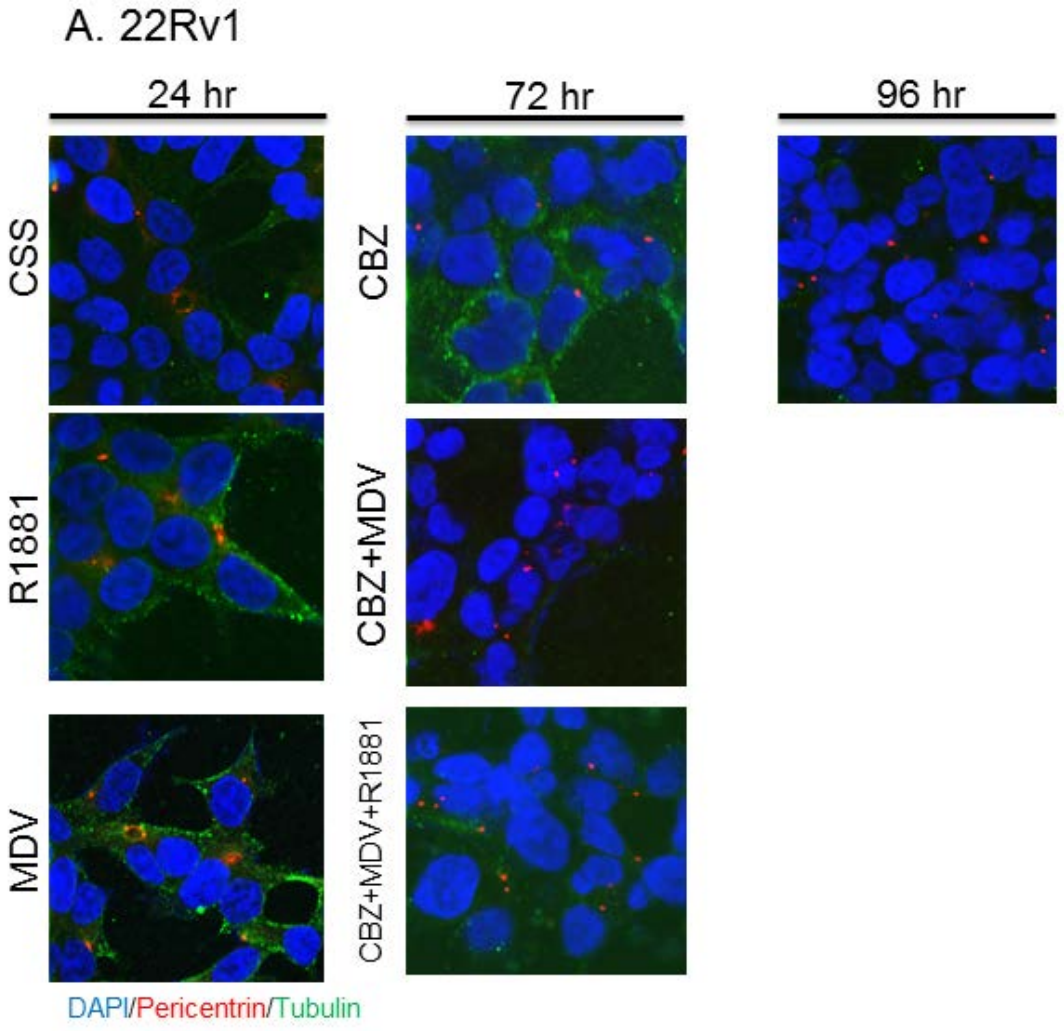


Figure 3.9. In Vivo Therapeutic Regimen of Cabazitaxel in a Transgenic Mouse Model of Aggressive Prostate Tumor Progression. Panel A, Schematic depiction of CBZ/VHC treatment administration to TRAMP+DNTGFβRII transgenic mice utilizing highest non-toxic therapeutic dose over a course of 14 days. Panel B, Scheduling of castration and CBZ/VHC treatment regime in TRAMP+DNTGFβRII transgenic mice utilizing highest non-toxic therapeutic dose.

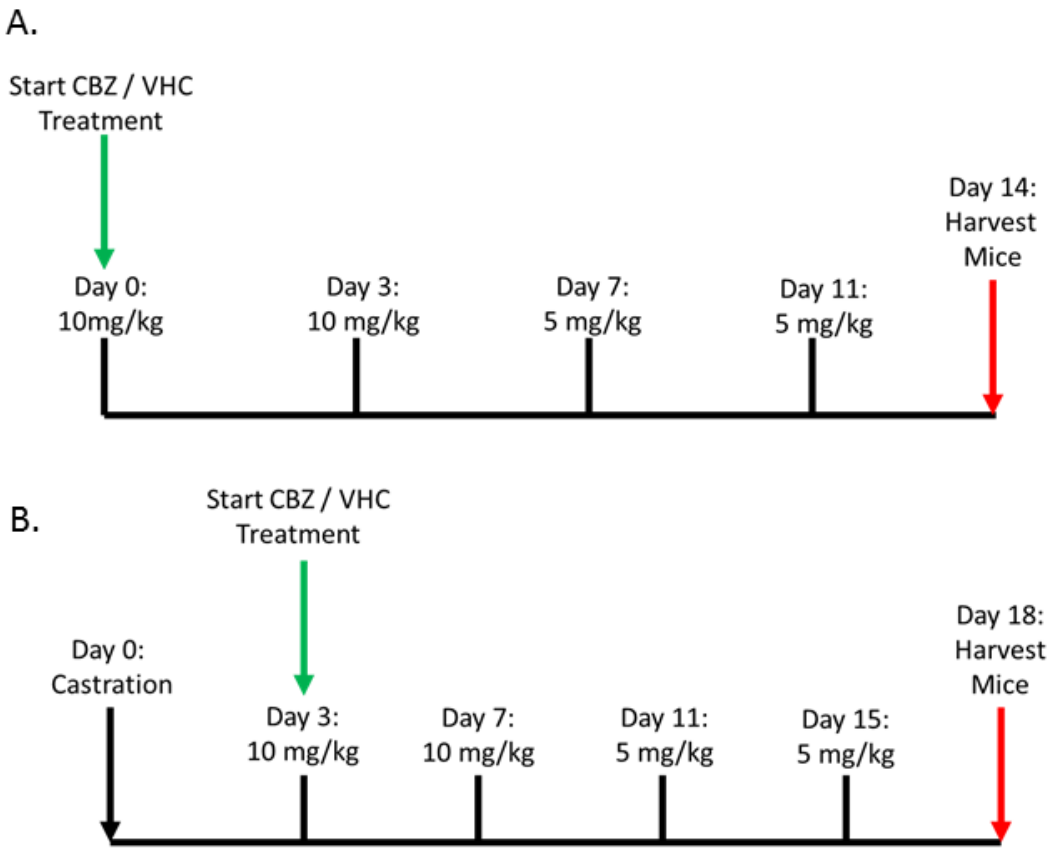
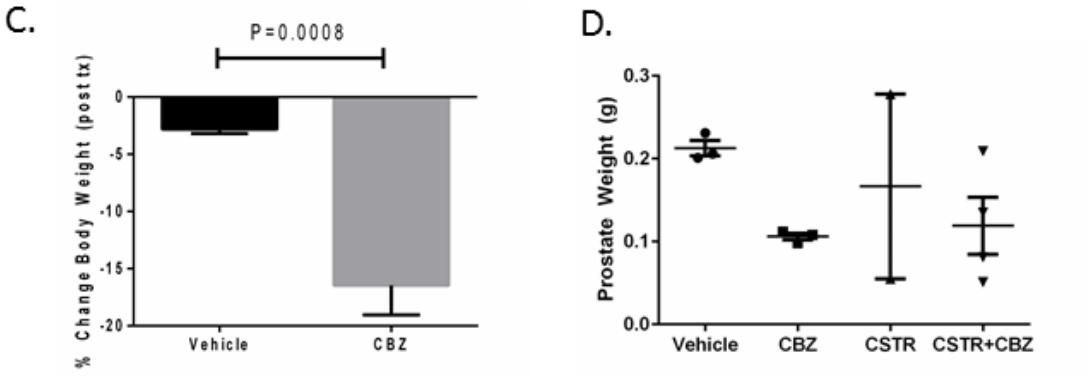


Figure 3.9 continued, In Vivo Therapeutic Regimen of Cabazitaxel in a Transgenic Mouse Model of Aggressive Prostate Tumor Progression. Panel C, The relative % change in mouse body weight on day-14 of CBZ treatment vs. VHC controls compared to body weight at experiment start. Data represent the mean of percent body weight change of all animals ± SEM; * indicates P<0.05 as determined Student's t-test. Panel D, Effect of CBZ, CSTR, and CSTR+CBZ on prostate gland weight in transgenic mice.



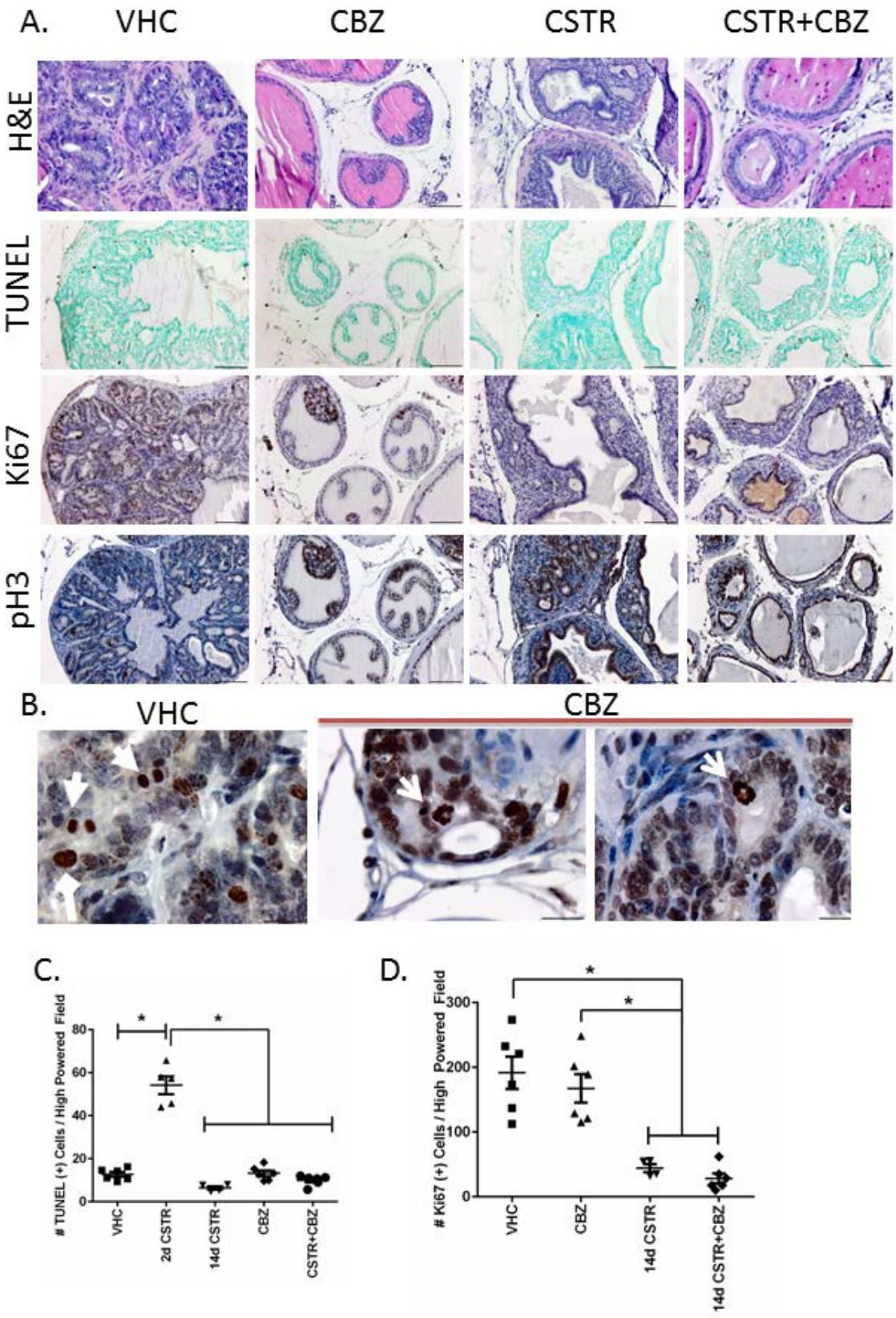

A.
VHC
CBZ
CSTR
CSTR+CBZ
H&E
TUNEL
Ki67
pH3
B.
VHC
CBZ
C.
TUNEL (+) Cells / High Powered Field
0
20
40
60
80
VHC
2d CSTR
14d CSTR
CBZ
CSTR+CBZ
*
*
D.
Ki67 (+) Cells / High Powered Field
0
100
200
300
VHC
CBZ
14d CSTR
14d CSTR+CBZ
*
*

Figure 3.10. *In Vivo* Effect of Cabazitaxel on an Androgen-Responsive Prostate Cancer Model of Tumor Progression to Lethal Disease. Panel A, Histopathological appearance of prostate tumors from transgenic mouse model of prostate tumor progression TRAMP/DNTGFβRII (400x magnification, scale bars are 50 μM). Tumors from control mice (VHC), castrated for 14 days (CSTR), or treated with CBZ for 14 days alone (CBZ) or in combination with castration induced androgen depletion (CSTR+CBZ) were subjected to immunostaining analysis. Serial sections were used for apoptosis detection (TUNEL) and cell proliferation (Ki-67 and phospo-H3 nuclear staining). Panel B, immunostaining for phospho-histone H3 expression identifying endoreduplication of nuclei in prostate tumor cells from the transgenic model of TRAMP/DNTβRII of advanced prostate cancer after 2wks of CBZ treatment; visualized under 100x oil immersion objective, scale bars are 50 μM. Panel C, Quantitation of apoptosis analysis of TUNEL-positive cells. Panel D, Quantitative analysis of cell proliferation in prostate tumors from vehicle control and treated mice. The number of positive cells per high power field was counted and data represent the mean of three non-continuous fields counted by two independent reviewers (N.K. and S.K.M.) ± SEM; * indicates $P<0.05$ as determined by one way ANOVA.

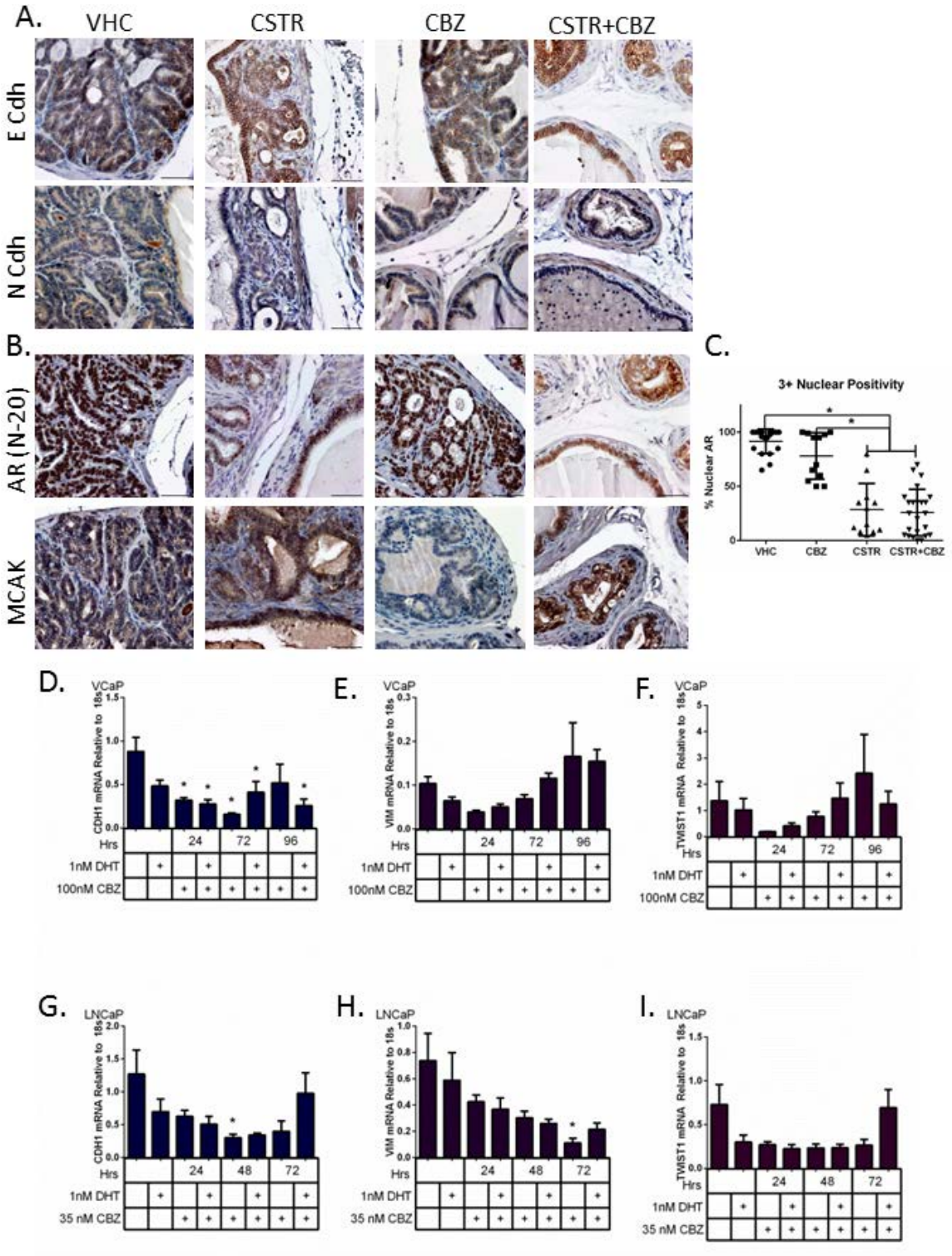

A.
VHC
CSTR
CBZ
CSTR+CBZ
E Cdh
N Cdh
B.
AR (N-20)
MCAK
C.
3+ Nuclear Positivity
% Nuclear AR
VHC
CBZ
CSTR
CSTR+CBZ
D.
VCaP
CDH1 mRNA Relative to 18s
Hrs
1nM DHT
100nM CBZ
E.
VCaP
VIM mRNA Relative to 18s
F.
VCaP
TWIST1 mRNA Relative to 18s
G.
LNCaP
CDH1 mRNA Relative to 18s
35 nM CBZ
H.
LNCaP
VIM mRNA Relative to 18s
I.
LNCaP
TWIST1 mRNA Relative to 18s

Figure 3.11: Cabazitaxel Impairs Advanced Prostate Cancer by Inducing MET and Targeting Kinesins. Panel A & B, Immunoreactivity profile of E-cadherin, N-cadherin, AR and MCAK expression in prostate tumor sections from control (VHC), castrated (CSTR), Cabazitaxel-treated (CBZ) or castration and Cabazitaxel (CSTR+CBZ) treated TRAMP/DNTGFβRII transgenic mice. CBZ induces EMT phenotypic changes and reduces AR (without affecting nuclear localization) and kinesin expression. Magnification 400x; scale bars are 50 μM. Panel C Quantification of AR nuclear staining (3+) in respective sections as indicated; numerical data (expressed as % nuclear AR) represent the mean of three non-continuous fields ± SEM; * indicates $P<0.05$ as determined by one way ANOVA. Panels D, E and F, RT-PCR analysis of mRNA expression for EMT effectors, E-cadherin (CDH1), Vimentin (VIM) and Twist1 (TWIST1) in VCaP cells after CBZ treatment in the presence or absence of androgens (24, 72 and 96hrs). Panels G, H and I, results of RT-PCR mRNA profiling of EMT genes in LNCaP cells in response to CBZ. Data represent the mean of three independent experiments analyzed in duplicate ± SEM; *indicates $P<0.05$ as determined by multiple comparisons of two way ANOVA.

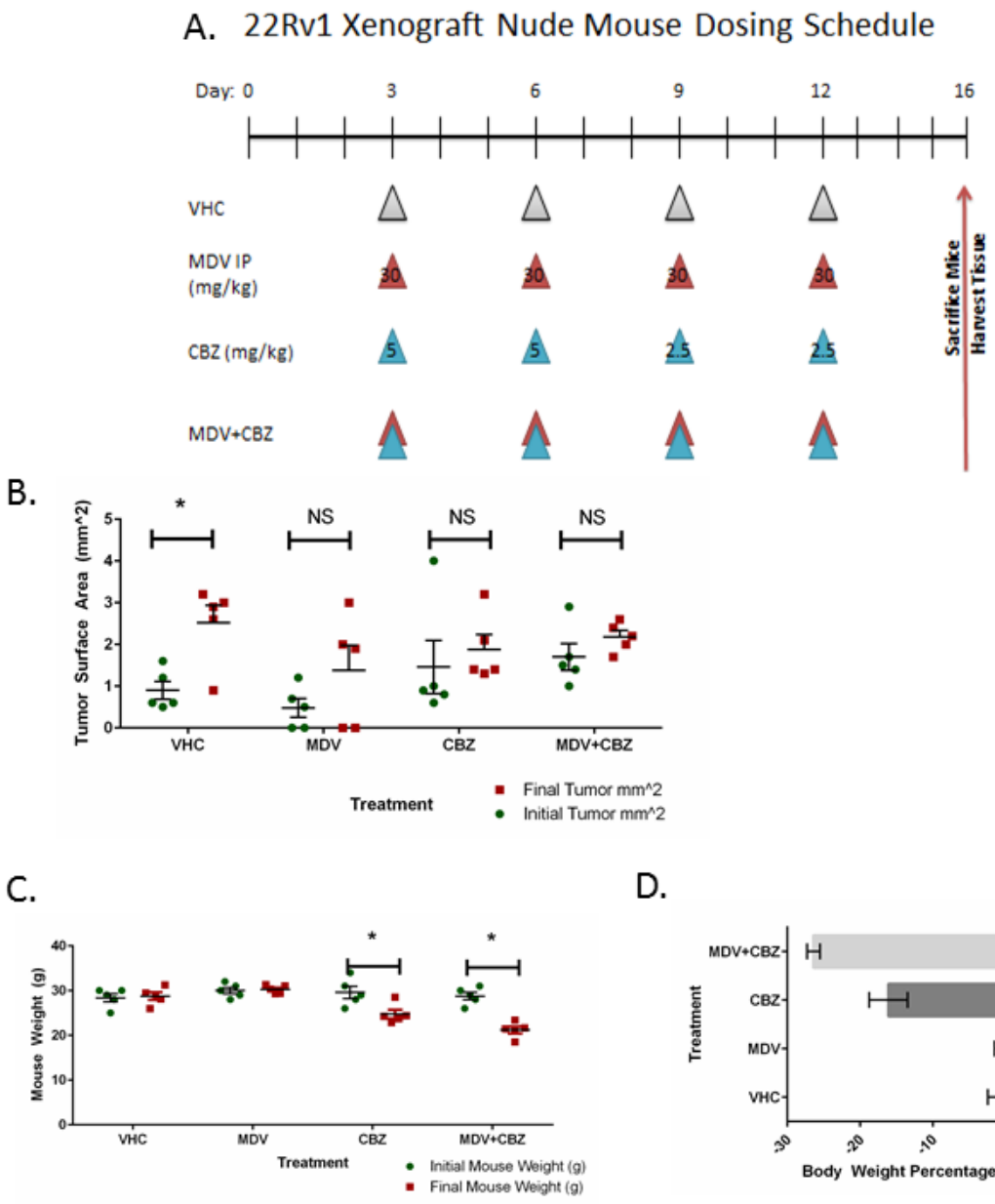

A. 22Rv1 Xenograft Nude Mouse Dosing Schedule
Day: 0
3
6
9
12
16
VHC
MDV IP (mg/kg)
30
CBZ (mg/kg)
5
2.5
MDV+CBZ
Sacrifice Mice
Harvest Tissue
B.
Tumor Surface Area (mm^2)
*
NS
NS
NS
VHC
MDV
CBZ
MDV+CBZ
Treatment
Final Tumor mm^2
Initial Tumor mm^2
C.
Mouse Weight (g)
*
*
VHC
MDV
CBZ
MDV+CBZ
Treatment
Initial Mouse Weight (g)
Final Mouse Weight (g)
D.
Treatment
MDV+CBZ
CBZ
MDV
VHC
Body Weight Percentage Change

Figure 3.12. Therapeutic Dosing Regimen of Cabazitaxel Treatment in Nude Mice Bearing 22Rv1 CRPC Xenografts. Panel A, Schematic depiction of dosing schedule in nude mice bearing subcutaneous tumors of 22Rv1 treated with MDV alone (30mg/kg), CBZ or the combination. Panel B shows the distribution of the tumor surface area in individual mice (n=5) at the initiation and termination of treatment (from Panel A). Panel C, Effect of treatment on mouse body weight. Data represent the mean of percent body weight change of all animals within respective treatment groups ± SEM; * indicates $P<0.05$ as determined Student's t-test. Panel D, The relative % change in mouse body weight on day-14 of CBZ treatment vs. VHC controls compared to body weight at experiment start.

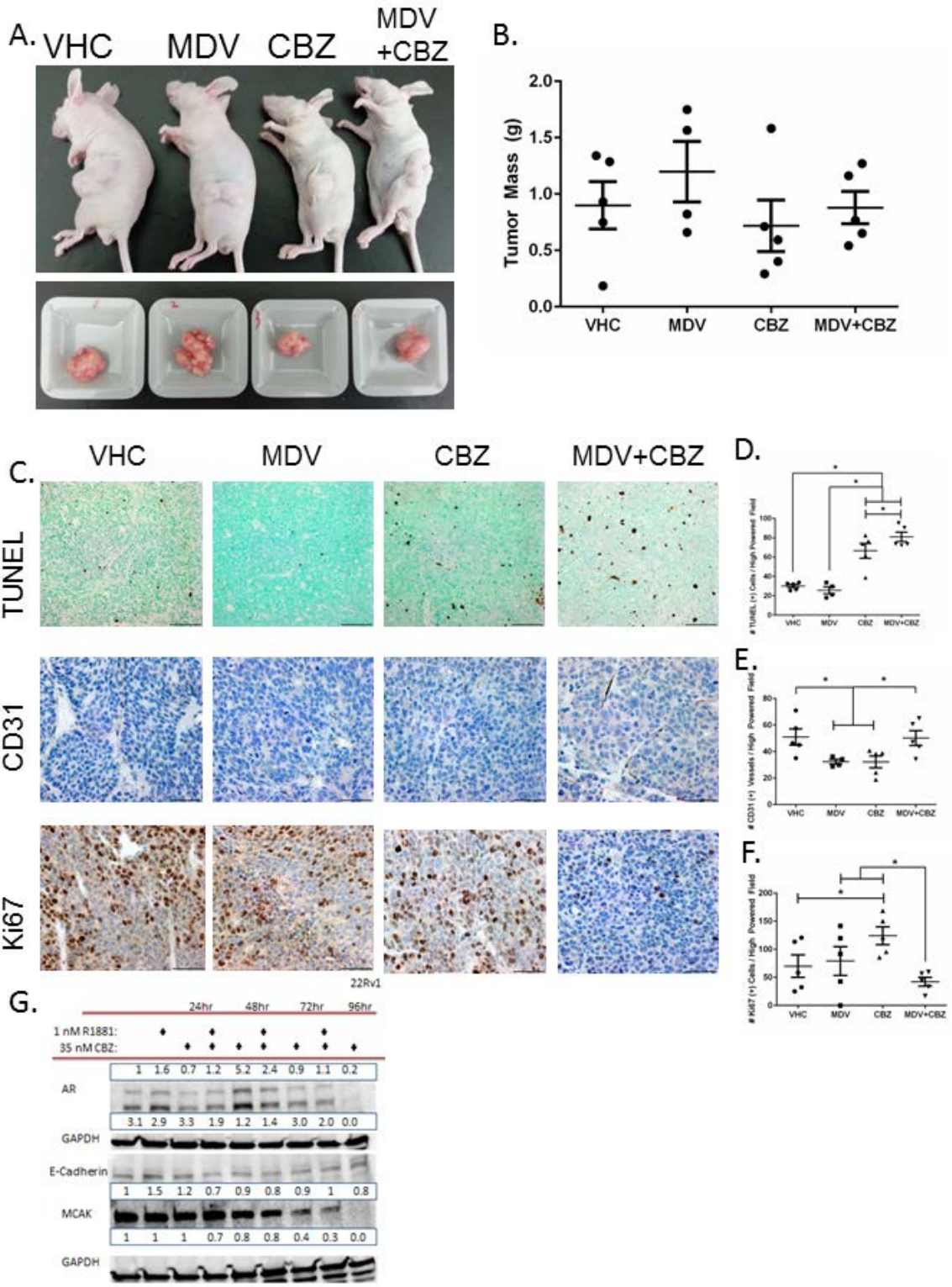
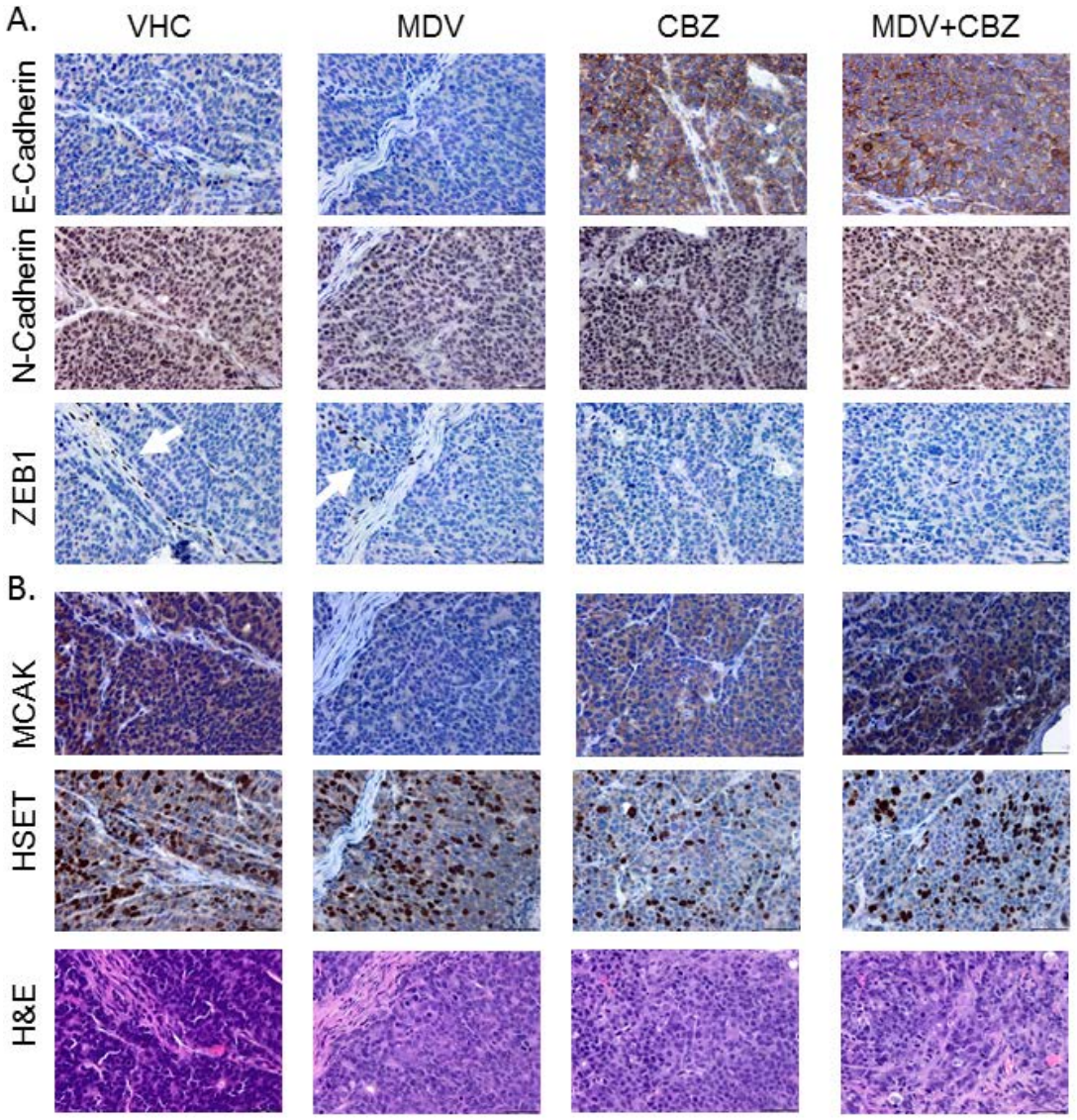

A.
VHC
MDV
CBZ
MDV +CBZ
B.
Tumor Mass (g)
2.0
1.5
1.0
0.5
0.0
VHC
MDV
CBZ
MDV+CBZ
C.
VHC
MDV
CBZ
MDV+CBZ
TUNEL
CD31
Ki67
22Rv1
D.
E.
F.
G.
24hr
48hr
72hr
96hr
1 nM R1881:
35 nM CBZ:
AR
1 1.6 0.7 1.2 5.2 2.4 0.9 1.1 0.2
3.1 2.9 3.3 1.9 1.2 1.4 3.0 2.0 0.0
GAPDH
E-Cadherin
1 1.5 1.2 0.7 0.9 0.8 0.9 1 0.8
MCAK
1 1 1 0.7 0.8 0.8 0.4 0.3 0.0
GAPDH

Figure 3.13. Effect of Cabazitaxel and Antiandrogen (Enzalutamide) on CRPC Xenograft Growth. Male nude mice were inoculated with CRPC 22Rv1 cells and when prostate tumors were palpable mice were exposed to vehicle (VHC), Cabazitaxel alone (CBZ), Enzalutamide (MDV) or combination (CBZ+MDV) for 2wks. Three days after the last treatment tumors were surgically excised and subjected to immunostaining analysis. Panel A, Characteristic images indicating gross appearance of prostate tumors after various treatments. Panel B, indicates the quantitative numerical data of tumor mass (g) of the 22Rv1 xenograft tumors in response to various treatments. Panel C, reveals immunohistochemical assessment of serial sections of 22Rv1 tumor xenografts, for apoptosis (TUNEL), CD31 (vascularity) and cell proliferation (Ki-67); scale bars are 50 µM. Panels D, E and F, Quantitative analysis of the numerical data for the TUNEL-based detection of apoptosis incidence, CD31-detected vascularity and Ki-67–based proliferative index, respectively. The number of positive cells per high power field was counted and data represent the mean of three non-continuous fields counted by two independent reviewers ± SEM; * indicates $P<0.05$ as determined by one way ANOVA. Panel G, Western blot analysis of AR (N-20), kinesin MCAK and E-cadherin (EMT) in 22Rv1 cells treated with CBZ (35nM) alone, or in the presence of androgens (1nM R1881) for 24-96hrs. GAPDH was used as loading control. Numerical data indicate the relative expression based on densitometric analysis.

Figure 3.14. Cabazitaxel Reverses EMT and Retains Nuclear AR in Human CRPC Xenograft. Panel A, Profiling of the EMT landscape in 22Rv1 prostate xenografts in response to CBZ and antiandrogen (MDV) treatment as single agents or as combination regime. Serial sections from control (VHC) and treated tumor-bearing mice were subjected to immunostaining for protein markers, E-cadherin, N-cadherin and Zeb-1; Magnification 400x, scale bars are 50 µM. Panel B, Effect of CBZ treatment on kinesin immunoreactivity, for MCAK and HSET proteins. Lower panel indicates H&E staining of serial sections of CRPC tumors.



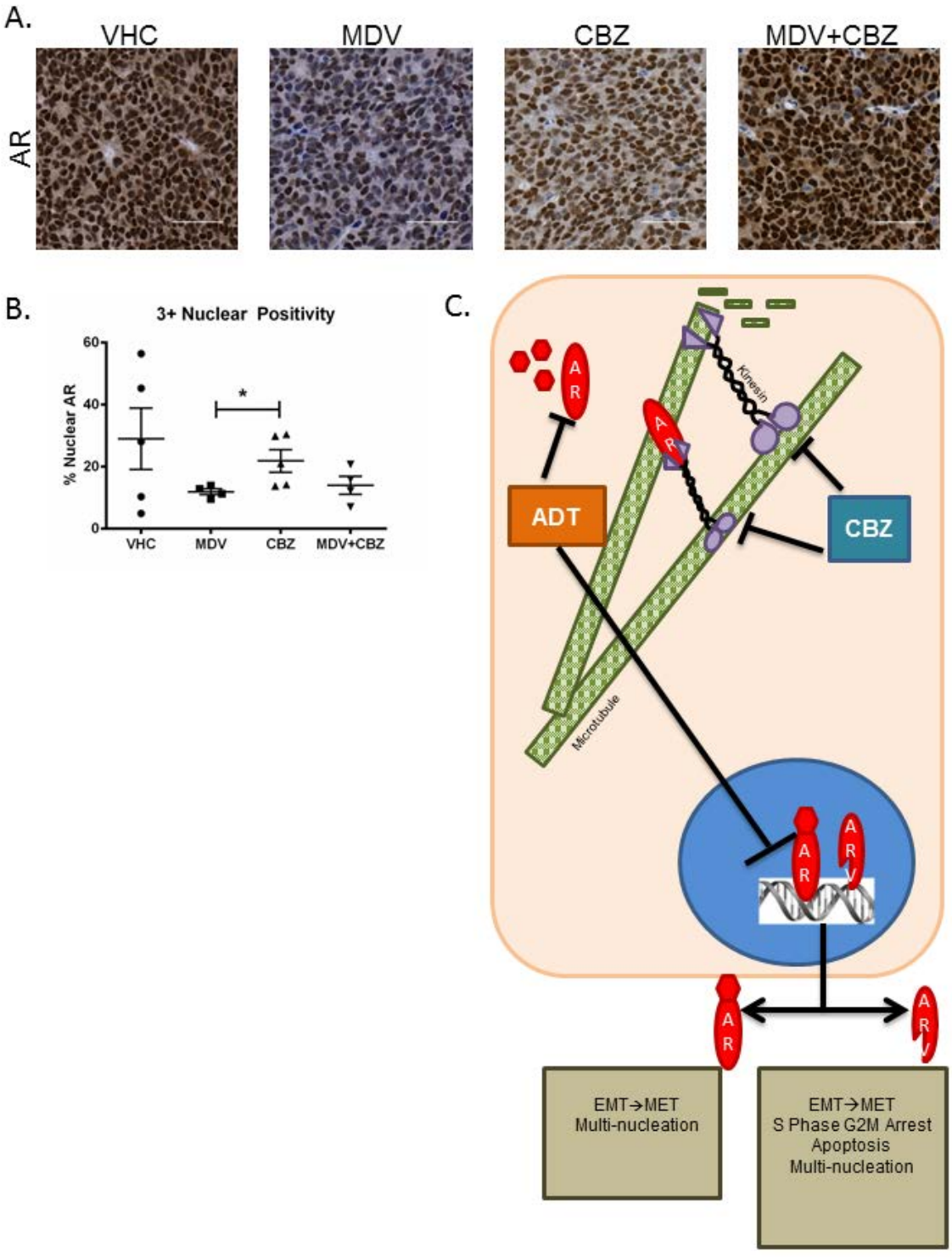

A.
VHC
MDV
CBZ
MDV+CBZ
AR
B.
3+ Nuclear Positivity
% Nuclear AR
60
40
20
0
*
VHC
MDV
CBZ
MDV+CBZ
C.
AR
ADT
CBZ
Kinesin
Microtubule
ARV
EMT→MET
Multi-nucleation
EMT→MET
S Phase G2M Arrest
Apoptosis
Multi-nucleation

Figure 3.15: Therapeutic Action of Cabazitaxel by Targeting AR and Mitotic Kinesins in CRPC. Panel A, CRPC prostate 22Rv1 tumors from VHC control and after treatment with MDV3100, Cabazitaxel as single agents or in combination were subjected to AR (N-20) immunostaining. Magnification 400x; scale bars are 50 µM. Cabazitaxel reduces AR expression but retains AR nuclear localization compared to VHC. Panel B, Data represent the mean of quantitated distribution of AR (N-20) in response to various treatments ± SEM; * indicates $P<0.05$ as determined by one way ANOVA. Panel C, Schematic illustration of targeting actions of CBZ against AR (independent of status) and Kinesins leading to EMT reversal and apoptosis. CBZ inhibits kinesin expression (at protein and mRNA level) and reduces AR expression and activity (full length and variants). The inhibitory effect by Cabazitaxel on mitotic kinesins across the microtubules however compromises export of AR from the nucleus into the cytosol, retaining nuclear AR localization and thus conferring therapeutic resistance. Combination of Cabazitaxel with androgen deprivation therapy (ADT) causes cytosolic distribution of AR (full length variants) towards overcoming cross-resistance. Therapeutic response to Cabazitaxel in advanced prostate cancer proceeds via novel action in causing phenotypic reversal of EMT to MET and multi-nucleation, (in addition to G2M + S phase arrest and apoptosis induction).

Chapter 4. Discussion

Taxane-based chemotherapy is an effective treatment for CRPC via stabilization of microtubules. Previous studies have demonstrated that microtubule-stabilizing chemotherapeutic agents such as Docetaxel and Paclitaxel inhibit AR nuclear localization and activity in human prostate cancer, an action that correlates with the therapeutic response (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010a). Moreover, the interaction between AR and tubulin occurs via binding in the N-terminal domain of AR (M.-L. Zhu et al., 2010a). My initial studies investigated the anti-tumor efficacy of a combination of AR N-terminal targeting EPI with Docetaxel chemotherapy in models of prostate cancer (Chapter 2). I found that there was no significant effect on the androgen-mediated nuclear transport of AR variants and AR transcriptional activity by Docetaxel. Given that 22Rv1 cells the full-length AR as well as the AR splice variants, this result was not entirely unexpected. These results gain mechanistic support from recent evidence by Chan and colleagues demonstrating that AR splice variants activate AR target genes and promote prostate cancer growth independent of canonical AR nuclear localization signal (S.C. Chan et al., 2012). More recently, it was reported that nuclear translocation and transcriptional activity of AR V7 variant was not affected by microtubule-targeting chemotherapy while ARv567es could associate significantly with microtubules and be modulated as such by taxanes (Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, Plymate, et al., 2014). Treatment of CRPC with EPI leads to decreased transcriptional activity of both full-length AR and constitutively active truncated variants (Andersen et al., 2010; Myung et al., 2013). Further understanding of the mechanisms of aberrant activation of AR and the distinct transcriptome of AR variants compared to full length wild-type AR, is central to the optimization of therapeutic targeting of CRPC and of major significance in patients who failed Docetaxel therapy.

The therapeutic response to Docetaxel was greatly enhanced by inhibition of the NTD of AR (by EPI) through cycling of EMT to MET among prostate cancer epithelial cells. These results support that transient "programming" of EMT by the AR NTD inhibitor, potentially drives the sensitivity of prostate tumors with differential distribution of AR variants to microtubule-targeting chemotherapy. My work provided the first evidence that combination of the microtubule-targeting agent, Docetaxel with targeting the AR NTD with EPI leads to enhanced anti-tumor action possibly via changes in the EMT landscape and the cytoskeleton of prostate cancer cells harboring AR variants. A functional significance of the EMT process in therapeutic response to microtubule-targeting is supported by the recently reported association between reduced E-cadherin expression and Docetaxel-resistance in prostate cancer cells (Puhr et al., 2012). Reduced E-cadherin expression promotes loss of cell adhesion, cell polarization, and gain of cell migration, which leads to invasion and metastases (J. Yang & R. A. Weinberg, 2008; Yilmaz & Christophori, 2009). Moreover, there is growing clinical evidence implicating EMT as a cellular mechanism conferring therapeutic resistance, development of metastases, and contributing to patient mortality (Puhr et al., 2012). Considering that interference with the androgen / AR signaling axis is associated with EMT induction (Matuszak & Kyprianou, 2011b; Y. Sun et al., 2011; M. Zhu & Kyprianou, 2010), Docetaxel administration prior to androgen deprivation with EPI may result in improved therapeutic outcomes via navigating the EMT-MET cycling, towards cytoskeleton remodeling and sensitizing prostate tumor cells to androgen- depletion mediated apoptosis. An alternative mechanistic scenario is that mutations in tubulin may affect the binding sites of taxanes leading to therapeutic resistance (Giannakakou et al., 1997; Orr et al., 2003). One may argue that when taxanes are structurally unable to effectively bind tubulin, the microtubules can no longer become stabilized, an effect that

fails to elicit disruption of cell cycle progression, and consequentially the AR is free for a nuclear translocation (schematically illustrated on Figure. 2.8.D).

In subsequent studies (Chapter 3), I focused on the anti-tumor action of Cabazitaxel, a 2nd line chemotherapy in models of CRPC. My results provided intriguing new insights into the ability of Cabazitaxel not to block AR translocation to the nucleus, but to reduce AR expression, both *in vitro* and *in vivo* models of androgen sensitive as well as CRPC. These observations are in sharp contrast to the effect Docetaxel has on inhibiting nuclear translocation of the AR to the nucleus in clinical prostate cancer (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; M.-L. Zhu et al., 2010b). Moreover, the response and sensitivity of human prostate cancer cells to Cabazitaxel was not associated with AR expression status. Thus PC3v567es (AR variant) and VCaP (AR full length) exhibited similar resistance to Cabazitaxel treatment in prostate cancer cells. These findings resonate with recent evidence indicating that the therapeutic anti-tumor effect of Cabazitaxel proceeded via an AR-independent mechanism (van Soest et al, 2014). Until recently, the mechanisms driving the therapeutic cross-resistance to taxanes and anti-androgens in CRPC included microtubule stabilization, AR expression and activity, and decreased nuclear localization of AR by interfering with tubulin-AR association via dynein (Darshan, Loftus, Thadani-Mulero, Levy, Escuin, Zhou, et al., 2011; Mistry & Oh, 2013; M.-L. Zhu et al., 2010a). It was surprising that Cabazitaxel would not inhibit AR nuclear translocation similarly to Docetaxel and actually promote nuclear accumulation, but further that translocation of ARv567es would not exhibit a similar pattern of nuclear accumulation like that seen for full length AR. PC3v567es cells, harboring the AR variant, demonstrated cytoplasmic accumulation of AR in presence of Cabazitaxel which was over ridden by androgen treatment.

Compelling mechanistic evidence from recent studies supports two new signaling effectors conferring resistance to taxane chemotherapy in CRPC, ERG over expression (Galetti et al, 2014) and the GATA2-IGF2 axis (Vidal et al, 2015). ERG overexpression represents one potential contributing explanation for Cabazitaxel resistance among prostate cancer cell lines investigated herein. It has been shown that TMPRSS2:ERG gene fusions lead to over expression of the transcription factor ERG, but most recently it has also been shown that ERG over expression can induce microtubule bending which prevents taxanes from efficiently binding and stabilizing β-tubulin subunits within the microtubule structure (Galletti et al., 2014; Perner et al., 2006). PC3, LNCaP, DU145, and 22Rv1 human prostate cancer cell lines do not possess TMPRSS2:ERG or TMRPSS2:ETV1 fusions, but the androgen sensitive prostate cancer line VCaP has significant copy number gain of ERG on chromosome 21 (Perner et al., 2006).

In accordance with existing evidence on the action of this taxane, I found that Cabazitaxel can effectively stabilize microtubule structures in androgen-sensitive and castration-resistant prostate cancer cells (Azarenko et al., 2014; Vrignaud et al., 2013). Cabazitaxel was designed to bind with β-tubulin subunits and stabilize their interaction preventing de-polymerization of the microtubule structure. Once assembled, microtubules cannot disassemble leading to mitotic blockade. However in contrast to Docetaxel that induced microtubule stabilization leading to classic G2M arrest and apoptosis as well as preventing AR nuclear import, I report for the first time that Cabazitaxel treatment can induce severe multi-nucleation, promote centrosome clustering, and lead to the development of mono-astral spindle formation (Nakouzi et al., 2014). These aberrations of mitosis lent insight that in addition to microtubule stabilization, Cabazitaxel treatment could be modulating the function of the mitotic kinesins which facilitate this process. Centrosome amplification promotes transient

spindle multi-polarity during mitosis and correlates with tumor aggressiveness (Ogden et al., 2014). During cell migration in interphase, centrosome-mediated nucleation of a microtubule array contributes to a formation of polarized Golgi apparatus, to enable directionality. The biological events engaged by cancer cells to navigate their supernumerary centrosomes towards directionality during cell migration are not fully understood but their potential therapeutic targeting by microtubule-targeting chemotherapy such as Cabazitaxel appears promising. The present study supports such a notion in CRPC.

In my efforts to understand why Cabazitaxel would fail to prevent AR nuclear translocation and result in a severe endoreduplication phenotype in prostate cancer cells, I identified the mitotic centromere-associated kinesin (MCAK) and HSET as targets of Cabazitaxel. MCAK is a member of the kinesin-13 subfamily and is a non-motile, microtubule depolymerizing kinesin that targets to microtubule tips and utilizes its' ATP hydrolysis power stroke to "flick" tubulin subunits off the end of the structure during mitotic progression (Desai et al., 1999; Ogawa et al., 2004; Rath & Kozielski, 2012). HSET is a member of the Kinesin-14 subfamily and like the rest of this subfamily is a "minus" end directed motor protein unlike the rest of the Kinesins. HSET promotes proper bi-spindle pole formation and facilitates proper cytokinesis. Furthermore, HSET has been shown to be overexpressed in Docetaxel resistant tumors (De et al., 2009). High expression of HSET has been strongly correlated with metastasis in non-small cell lung cancer to the brain (Watts et al., 2013). HSET is an attractive cancer target because its' downregulation does not have any negative consequences in non-malignant cells, while interruption of centrosomal clustering is catastrophic for cancer cells with supernumerary chromosomes (Watts et al., 2013).

The inhibitory effect of Cabazitaxel on protein and mRNA expression of MCAK and HSET kinesins provides the first evidence that a microtubule targeting chemotherapy can effectively target a mitotic kinesin towards tumor suppression. Significantly enough, clinical evidence documented that progression to CRPC in patients is correlated with overexpression of MCAK (Sircar et al., 2012). This association resonated with our findings in the pre-clinical model of prostate cancer progression (Chapter 3). Another kinesin, the kinesin spindle protein (KSP) is a molecular motor that crawls along the microtubules to facilitate cell division (also known as Eg5 or KIF11). Although an inhibitor specific to Eg5 (KSP) has been evaluated in Phase II clinical trials for relapsed multiple myeloma, the clinical data indicate that such KSP inhibitors (Ispinesip) might be efficacious in combination with proteasomal inhibitors or immuno-modulators as frontline therapy for myeloma patients. In prostate cancer, early-phase clinical trials in CRPC patients using the first generation Eg5 inhibitor, Ispinesip, have met only limited success (Blagden et al, 2008; Beer et al; 2008). Eg5 is functionally involved in prostate cancer cell growth *in vitro* and *in vivo* models (Hayashi et al, 2008; Davis et al; 2006). In accord with our present findings, MCAK was identified as a mitosis phase target in prostate cancer that was overexpressed in CRPC gene expression datasets and is associated with disease progression as well as taxanes resistance (Ganguly et al., 2011; Sircar et al., 2012). This work suggests that Cabazitaxel chemotherapy targets expression of these mitotic kinesins (MCAK and HSET) which confer resistance to Docetaxel chemotherapy, and this evidence is of high mechanistic significance as it provides a new molecular platform for Cabazitaxel to impart additional increase in survival where Docetaxel chemotherapy fails.

Targeting the process of EMT by phenotypic reversion to MET in the context of the tumor microenvironment acquires an attractive therapeutic value in metastatic

tumors. Utilizing a transgenic mouse model of prostate cancer progression driven by EMT induction, I analyzed the consequences of Cabazitaxel on EMT in prostate tumor progression *in vivo*. At 18-20wks of age our transgenic mouse model is characterized by aggressive and poorly differentiated prostate tumors (Pu et al 2009). The paradoxical observation was made that prostate tumors from mice treated with Cabazitaxel alone or in combination with castration-induced androgen depletion, exhibited a phenotypically differentiated prostate epithelium with intact luminal secretions. One could argue that the plasticity afforded to a fully differentiated epithelium by Cabazitaxel treatment, allows individual cells to de-differentiate into mesenchymal-like derivatives in reversible phenotypic transformative process. Thus during several rounds of EMT and the reverse process, MET, allow for the formation of well-differential glandular epithelial structures in response to Cabazitaxel. This is supported indirectly by the observation that the incidence of apoptosis in prostate tumors in response to Cabazitaxel, or the combination treatment of Cabazitaxel and castration was significantly lower compared to high apoptosis induced by castration-induced androgen deprivation. In an interesting twist of growth kinetics, Cabazitaxel increased the proliferative activity of prostate tumor cell populations compared to castrated mice and was similar to the intact control mice. Considering that a dynamic EMT-MET cycling has been functionally implicated in the formation of complex epithelial tissues (Moreno-Bueno et al., 2008), my data strongly support the ability of Cabazitaxel to induce phenotypic glandular formation of CRPC advanced prostate tumors via reversal of EMT. Thus, I speculate that upon elimination of the primary population of prostate cancer cells targeted by Cabazitaxel and androgen-depletion, a subset population of prostate tumor epithelial cells undergo MET (EMT reversal) in response to treatment, reversing the tumorigenic phenotype to well-differentiated epithelial cells and potentially overcoming phenotypic resistance. This work gains support from recent evidence suggesting that resistance to Cabazitaxel

chemotherapy can be driven by EMT (Figure 1.5.G) (Duran et al., 2014). Using gene expression profiling techniques it was identified that Cabazitaxel resistant tumor cells possessed alterations in the expression of EMT marker profiles. Specifically, they demonstrated increased expression of the mesenchymal marker Vimentin and decreased expression of the epithelial marker E-cadherin compared to parental controls (Duran et al., 2014). Similar effects were documented on Vimentin, E-Cadherin and Twist mRNA expression patterns in VCaP cell lines (Cabazitaxel resistant) compared to LNCaP (Cabazitaxel sensitive). Thus, Cabazitaxel treatment can induce EMT←→ MET cycling which may confer resistance to some prostate cancer cells and may also initiate programming to generate a phenotypically differentiated prostate epithelium with intact luminal secretions. In Chapter 2 it was described that EPI treatment induced EMT ←→MET cycling which sensitized CRPC xenografts to Docetaxel treatment (Figure 3.15). Here, it is demonstrated that Cabazitaxel resistance can be conferred by EMT transcriptional programming highlighting the differences between Docetaxel and Cabazitaxel.

The findings presented herein provide a molecular basis for the emerging role of AR variants in predicting therapeutic resistance of advanced CRPC to Enzalutamide (Antonarakis et al., 2014) and taxanes in experimental models of CRPC (Thadani-Mulero, Portella, Sun, Sung, Matov, Vessella, Corey, Nanus, Plymate, et al., 2014). Overexpression of AR splice variants may preclude patients from undergoing Cabazitaxel and anti-androgen (Enzalutamide, MDV) combination therapy due to potential therapeutic resistance. My results showed that combination of Cabazitaxel and MDV (at very high doses) leads to a surprising growth stimulatory response in the CRPC 22Rv1 cells *in vitro* and *in vivo*, but not in the androgen-sensitive VCaP cells (harboring full length AR) or androgen-independent PC3v567es. A molecular explanation for this

effect is provided by a very recent report indicating that MDV treatment of CRPC 22Rv1 cells has no effect on AR V7 variant expression, and actually upregulated V7 in VCaP cells (Schweizer et al., 2015). Attractive as the potential biomarker value of AR profiles might emerge in predicting clinical cross-resistance to Cabazitaxel and anti-androgens in CRPC patients, one must consider our present data pointing to the ability of Cabazitaxel to overcome phenotypic resistance in prostate tumors *in vivo* independently of AR variant status and nuclear localization. This points to a potential platform to optimize sequencing of these therapeutics, tailored to the unique expression profiles of AR and the molecular and phenotypic landscape of prostate tumors in individual CRPC patients.

This dissertation describes the first evidence that Cabazitaxel chemotherapy could impair prostate tumor progression to advanced disease by reversing EMT to MET and inducing phenotypically normal prostate luminal architecture in CRPC tumors. This novel taxane effect proceeds via a mechanism independent of AR variant expression status. My findings are of major therapeutic significance in providing an initial molecular insight into mechanisms of cross-resistance between Cabazitaxel and anti-androgens in patients with advanced mCPCR that will direct optimization of treatment sequencing for CRPC patients towards bypassing such resistance in advanced disease.

The goal of my dissertation work has been to investigate the mechanisms of taxane resistance in CRPC, I have utilized various therapeutic combinations to probe the mechanisms behind this resistance towards the sequencing of such strategies for effective clinical benefit in CRPC patients. The combination of the microtubule-targeting agent, Docetaxel with targeting the AR NTD with EPI leads to enhanced anti-tumor action possibly via changes in the EMT landscape and the cytoskeleton of prostate cancer cells harboring AR variants. Moreover, expression of AR variants in CRPC patients may contraindicate the use of combination of Cabazitaxel with MDV therapy,

and in such cases Cabazitaxel therapy alone would be more efficacious. In CRPC patient populations that are screened positive for AR V7 expression, consideration should be given for use of Cabazitaxel chemotherapy with intermittent EPI-002 treatment. As discussed earlier, AR V7 is an AR splice variant which lacks functional exons of the ligand binding domain causing it to become constitutively active; over expression of AR V7 confers therapeutic resistance to anti-androgens in patients with CRPC (Antonarakis et al., 2014). Where MDV fails to effectively target such constitutively active AR splice variants, EPI-002 binds the intrinsically disordered domain of the NTD preventing it from initiating transcription activation functions and DNA binding (Figure 1.3) (Andersen et al., 2010). EPI small molecule inhibitors bind and inhibit many of the AR variant isoforms expressed in advanced prostate cancer tumors such as ARV7 (Martin et al., 2014; Sadar, 2011). EPI can still effectively bind and inhibit activation of many AR variant isoforms as data generated in this thesis demonstrated (Martin et al., 2014). Combining the unique AR targeting features of EPI with the newly revealed capability of Cabazitaxel to decrease AR overall expression and modulate mitotic kinesin dynamics could offer an effective next generation targeting platform for Docetaxel - resistant CRPC patient cohorts with potential therapeutic impact.

There have been numerous trials investigating the use of mitotic inhibitors in combination with additional compounds with mixed results. Inhibition of Eg5 activates the spindle check point and induces mitotic arrest and apoptosis therefore, it is an attractive therapeutic target (Domenech & Malumbres, 2013). Of clinical significance is a recent retrospective study that identified Eg5 nuclear expression and a predictive biomarker of Docetaxel response in mCRPC patients and a prognostic biomarker for hormone naïve prostate cancer patients (Wissing et al., 2014). One such inhibitor, S-Trityl-L-Cysteine (STLC) exhibited an effect in Docetaxel-resistant prostate cancer cells

that was not impaired by P-Glycoprotein upregulation (Wiltshire et al., 2010). Furthermore, Eg5 inhibitors AZD4877, SB-715992, SB-743921, ARRY-520, ARQ621, LY2523355, and MK0731 have passed Phase I clinical trials as monotherapy (Domenech & Malumbres, 2013). Treatment with mitotic inhibitors have been well tolerated in patients and a Phase I trial investigating combination use of Eg5 inhibitor SB-715992 and Docetaxel in solid tumors is ongoing (NCT00169520) (Domenech & Malumbres, 2013). With the knowledge of the effect of Cabazitaxel on mitotic kinesin expression and apoptosis, promotion by preventing mitotic exit, it is tempting to consider exploitation of the combination of Eg5 inhibitors and Cabazitaxel therapies to overcome therapeutic resistance in the clinical disease (Domenech & Malumbres, 2013; Martin et al., 2015).

Finally, I would like to reflect on the potential impact of my work in prostate cancer. Recently, a lot of attention has surrounded the use of microtubule targeting therapeutics in Alzheimer's disease and other diseases in which neural regeneration is compromised (Lu, Lakonishok, & Gelfand, 2015). Specifically, the use of low dose vinblastine, another microtubule stabilizing therapeutic, has been shown to facilitate microtubule sliding, increase kinesin-mediated trafficking and enhance neurite repair in damaged *drosophila* neurons (Lu et al., 2015). While these results are extremely promising, the results of this study primarily focus on the activity of Kinesin 1, a traditional minus end directed, ATP-dependent motor protein. The focus of this highly publicized research was on vinblastine treatment but it is certainly possible that use of first-generation taxanes and even Cabazitaxel could be similarly worthy of investigation. Certainly, neurons do not undergo mitosis and targeting of mitotic kinesins such as MCAK and HSET by Cabazitaxel should not be disadvantageous for these populations. Future investigations could be directed to the identification of other members of the

kinesin superfamily that can be functionally impaired by Cabazitaxel treatment in diverse pre-clinical and clinical settings. Thus, tubulin targeting chemotherapy can be recognized as a multi-target approach in diseases beyond cancer.

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